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Alveolar lymphocyte proliferation induced by Propionibacterium acnes in sarcoidosis patients.

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Abstract

The proliferation of lymphocytes induced by Propionibacterium acnes (P. acnes) was measured by the in vitro incorporation of 3H-thymidine. The mean response rate of alveolar lymphocytes obtained by bronchoalveolar lavage was 2.23 +/- 0.89 in nine untreated sarcoidosis patients, 0.85 +/- 0.17 in five sarcoidosis patients given corticosteroids and 0.78 +/- 0.29 in 11 controls. The proliferation was significantly enhanced in the untreated patients compared to both the treated patients (p less than 0.01) and controls (p less than 0.001), but there was no significant difference in response rates between the treated patients and controls. The response rate of alveolar lymphocytes was significantly higher in four active patients (3.05 +/- 0.61) than in four inactive patients (1.77 +/- 0.44) (p less than 0.05) and in the controls (p less than 0.001). In sarcoidosis patients, the response rates showed a good correlation with activities of serum lysozyme (r = 0.695, p less than 0.01), and with percentages of lymphocytes in bronchoalveolar lavage fluid (r = 0.591, p less than 0.05). There was a low correlation between angiotensin-converting enzyme activities and the response rates (r = 0.508, p less than 0.1). Neither peripheral blood lymphocytes in sarcoidosis patients nor in controls showed any response to P. acnes, but alveolar lymphocytes of the untreated active sarcoidosis patients were sensitive to P. acnes. The lymphocytes activated by P. acnes may play a central role in the induction of alveolitis in sarcoidosis patients.

KEYWORDS: sarcoidosis, alveolar lymphocyte, lymphocyte proliferation, Propionibacterium acnes

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Alveolar Lymphocyte Proliferation Induced by *Propionibacterium acnes* in Sarcoidosis Patients

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The proliferation of lymphocytes induced by *Propionibacterium acnes* (*P. acnes*) was measured by the *in vitro* incorporation of ^{3}H-thymidine. The mean response rate of alveolar lymphocytes obtained by bronchoalveolar lavage was 2.23 ± 0.89 in nine untreated sarcoidosis patients, 0.85 ± 0.17 in five sarcoidosis patients given corticosteroids and 0.78 ± 0.29 in 11 controls. The proliferation was significantly enhanced in the untreated patients compared to both the treated patients ($p < 0.01$) and controls ($p < 0.001$), but there was no significant difference in response rates between the treated patients and controls. The response rate of alveolar lymphocytes was significantly higher in four active patients (3.05 ± 0.61) than in four inactive patients (1.77 ± 0.44) ($p < 0.05$) and in the controls ($p < 0.001$). In sarcoidosis patients, the response rates showed a good correlation with activities of serum lysozyme ($r = 0.695$, $p < 0.01$), and with percentages of lymphocytes in bronchoalveolar lavage fluid ($r = 0.591$, $p < 0.05$). There was a low correlation between angiotensin-converting enzyme activities and the response rates ($r = 0.508$, $p < 0.1$). Neither peripheral blood lymphocytes in sarcoidosis patients nor in controls showed any response to *P. acnes*, but alveolar lymphocytes of the untreated active sarcoidosis patients were sensitive to *P. acnes*. The lymphocytes activated by *P. acnes* may play a central role in the induction of alveolitis in sarcoidosis patients.

Key words: sarcoidosis, alveolar lymphocyte, lymphocyte proliferation, *Propionibacterium acnes*

Sarcoidosis is multiple organ disease characterized by the formation of epitheloid cell granulomas. The pathogenesis of sarcoidosis has been attributed to bacteria, fungi, mycoplasma organisms and chemical substances, but none of these agents has been substantiated. Homma, J. Y. and his co-workers reported that *Propionibacterium acnes* (*P. acnes*) was found in affected lymph nodes at higher frequencies and somewhat higher concentrations than in non-sarcoidosis samples(1, 2). Accordingly, they considered that *P. acnes* may be virulent and a causal pathogen of the disease.

Although most organs are attacked by the disease, the lungs are the first target organ (3). Pulmonary sarcoidosis is characterized by granulomas and alveolitis consisting primarily of lymphocytes and mononuclear phagocytes(4). According to recent findings that alveolitis in active sarcoidosis is accompanied by a marked increase in the number of activated T lymphocytes, it is thought that T lymphocytes play an important role in modulating the formation of granulomas (5-7). Thus, when studying the pathogenesis

of sarcoidosis, it is essential to understand the mechanism underlying the increase in the number of T lymphocytes and the activation of T lymphocytes within alveolar structures.

We hypothesized that the pathogen, which is aspirated through the respiratory tract to the alveolus and which activates alveolar lymphocytes, may be *P. acnes*. To evaluate this hypothesis, the proliferative responsiveness of alveolar lymphocytes to *P. acnes* in sarcoidosis patients was studied.

Materials and Methods

We studied 13 patients (eight males and five females). The diagnosis of sarcoidosis was made in 11 patients from consistent clinical pictures and biopsies showing epithelioid cell granulomas. and in two only from clinical pictures. The patients' ages ranged from 25 to 72 years (median, 45 years). The period of illness from the onset to the examination was 2 months to 18 years (median, 10 months). Three patients had bilateral lymphoadenopathy without parenchymal involvement (Stage I); six patients had lymphoadenopathy with parenchymal involvement (Stage II); three patients had parenchymal involvement without lympho-adenopathy (Stage III); and one patient had no evidence of involvement of the parenchyma and lymph nodes (Stage 0).

^{67}Ga-citrate scintigraphy showed positive uptake in the pulmonary parenchyma in six of eight untreated patients. Nine patients had no therapy and five patients were given corticosteroids at the time of examination. One patient was examined twice before and after corticosteroid therapy. The nine untreated patients were divided into three groups depending on the state of disease, which was judged from increased numbers of active T lymphocytes in bronchoalveolar lavage (BAL) fluid, ^{67}Ga-citrate scintigrams, elevation of serum angiotensin-converting enzyme (ACE) activity and chest roentgenograms. The disease was active in four patients, inactive in four and was not seen in one.

Nine healthy volunteers and two patients with lung cancer were used as controls. In the lung cancer patients, bronchoalveolar lavage was performed in sites of the lungs without cancer involvement.

Lavage was performed under endoscopic control with a fiberoptic bronchoscope. The fiberoptic bronchoscope was wedged into a subsegmental bronchus, and 50 ml of sterile normal saline was instilled via the suction channel of the bronchoscope and immediately aspirated into a collection chamber. The process was repeated four times. The fluid was filtered immediately through a stainless mesh to remove mucus, and centrifuged to separate mononuclear cells (MNC). The MNCs were washed three times, and the cell pellet was resuspended at a concentration of 1×10^6 lymphocytes per milliliter in RPMI 1640 with 10% fetal calf serum. Heparinized peripheral blood MNCs were separated by Conray-Ficoll density gradient centrifugation and suspended at a concentration of 1×10^6 lymphocytes per milliliter in RPMI 1640 medium.

P. acnes pyridine extract residue (PER) (*P. acnes*. strain 4142, RPMI, Immuno Chem Research Inc. MT, USA), at various concentrations from 0.5 μg/ml to 500 μg/ml, was added to each well. The cultures were incubated for 6 days at 37°C in a 5% CO_2 atmosphere, and 1 μCi of ^{3}H-thymidine was added to each well and cultivated for 7 more hours. Cultures were harvested on scintillation grade glass fiber strips using a multiple sample harvester, and incorporated radioactivity (dpm) was determined by counting in a liquid scintilator. The stimulation index was determined by the following formula;

$$\text{Stimulation index} = \frac{\text{dpm of lymphocytes treated with } P.\,acnes \text{ PER}}{\text{dpm of untreated lymphocytes}}$$

All experiments were done in triplicate.

Serum ACE activity was measured by a modification of the method of Kasahara, and serum lysozyme activity was measured by determining the initial rate of lysis by *Micrococcus lysdeikticus* suspended in the serum.

Results

Percentage of lymphocytes in bronchoalveolar lavage (BAL) fluid. The median percentage of lymphocytes in BAL fluid was 37.5% (ranging from 4.4% to 76.0%) in the

nine untreated sarcoidosis patients, 19.8% (ranging from 2.2% to 44.4%) in the five patients given corticosteroids and 6.6% (ranging from 4.9% to 14.6%) in the 11 controls.

Stimulation index of lymphocytes in BAL fluid induced by P. acnes PER. The mean percentage of stimulation indices of the nine untreated sarcoidosis patients was 1.37 at a concentration of *P. acnes* PER of 0.5 μg/ml, 1.80 at 1.0 μg/ml, 2.23 at 5.0 μg/ml, 2.06 at 10.0 μg/ml, 1.44 at 50 μg/ml, 1.00 at 100 μg/ml, and 0.81 at 500 μg/ml, showing a dose response curve with a peak at 5.0 μg/ml (Fig. 1). In the treated patients, the peak stimulation index (0.88) was obtained at a concentration of 5.0 μg/ml, but no significant differences were noted at various concentrations. In controls, the stimulation index was determined only at three concentrations of *P. acnes* because of the small number of lymphocytes in the BAL fluid. The mean value was 0.78 at 1.0 μg/ml, 0.78 at 5.0 μg/ml, and 0.84 at 10.0 μg/ml. There was no significant difference among the three groups, and the mean stimulation index was below 1.00 at all concentrations. The alveolar lymphocytes from controls showed no significant response to *P. acnes*.

Since the response curve of the untreated patients showed a peak at a concentration of 5.0 μg/ml, the stimulation index is discussed at this concentration hereinafter (Fig. 2). The mean stimulation index was greater in the nine untreated sarcoidosis patients (2.23 ± 0.89) than in the 11 control subjects (0.78 ± 0.29; $p < 0.001$) and the five treated sarcoidosis patients (0.89 ± 0.17; $p < 0.01$). However, no significant difference was noted between the treated patients and controls.

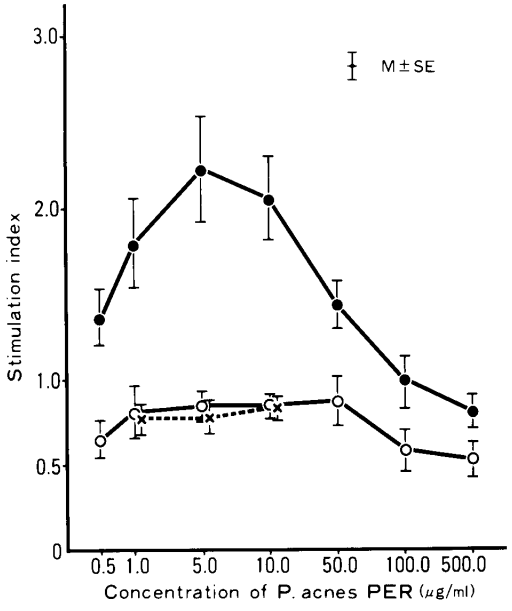

Fig. 1 Stimulation index of alveolar lymphocyte induced by *P. acnes* pyridine extract residue in sarcoidosis patients and controls. (○—○) With prednisolone; (●—●) Without prednisolone; (×····×) control.

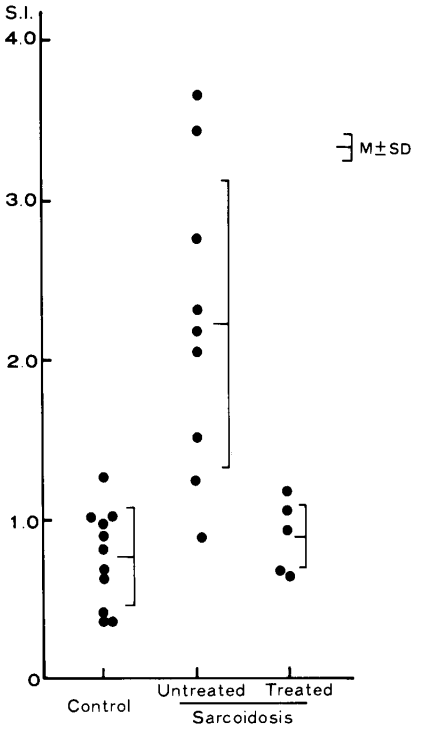

Fig. 2 Stimulation index of alveolar lymphocyte induced by *P. acnes* pyridine extract residue (5.0 μg/ml) in sarcoidosis patients and controls.

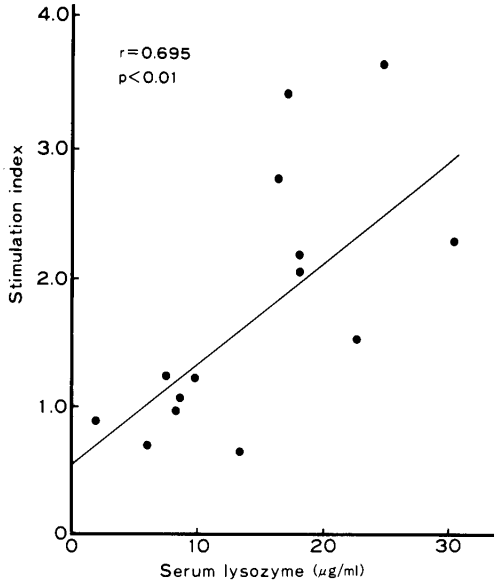

Fig. 3 Correlation between the stimulation index of alveolar lymphocytes induced by *P. acnes* and serum lysozyme activity in sarcoidosis patients.

The mean stimulation index was 3.05 ± 0.61 in the four active cases, 1.77 ± 0.44 in the four inactive cases and 0.89 in the resolved case. In the cases of active sarcoidosis, the stimulation index was significantly higher than in the controls ($p < 0.001$) and inactive sarcoidosis patients ($p < 0.05$). The mean stimulation index was 2.75 in two patients with Stage I, 2.74 in four patients with Stage II, 1.41 in two patients with Stage III, and 0.89 in the patients with Stage 0. It was significantly elevated in patients with Stage I and Stage II compared with that of controls.

The correlation between the stimulation index and various clinical data. In the untreated sarcoidosis patients, a good correlation was found to exist between the stimulation index and the serum lysozyme activity ($r = 0.695$, $p < 0.001$) (Fig. 3), and the percentage of lymphocytes in BAL fluid ($r = 0.591$, $p < 0.05$) (Fig. 4). In addition, there was a low correlation between the stimula-

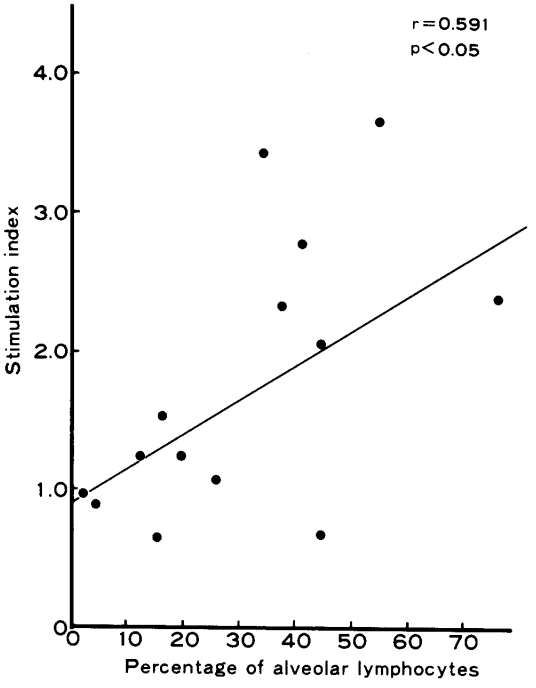

Fig. 4 Correlation between the stimulation index of alveolar lymphocytes induced by *P. acnes* and serum angiotensin-converting enzyme activity in sarcoidosis patients.

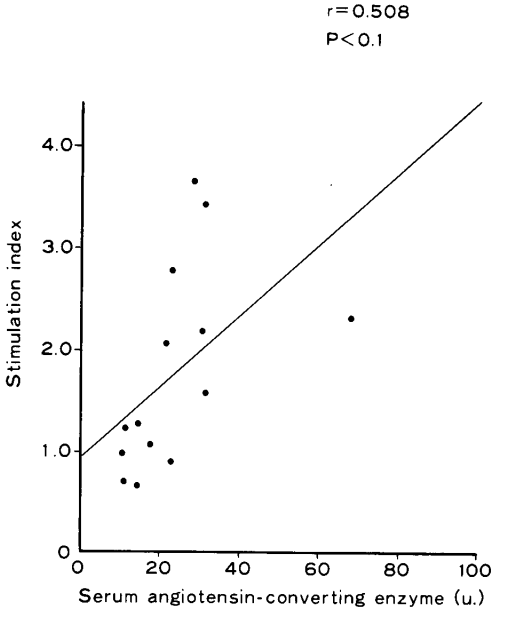

Fig. 5 Correlation between the stimulation index of alveolar lymphocytes induced by *P. acnes* and the percentage of lymphocytes in bronchoalveolar lavage fluid in sarcoidosis.

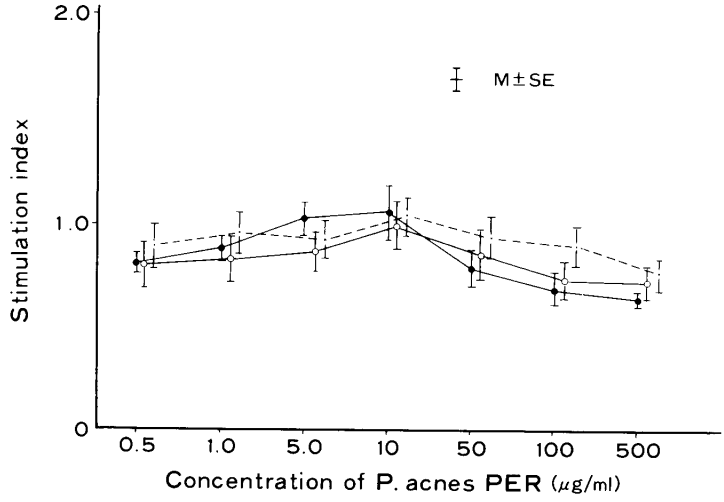

Fig. 6 Stimulation index of peripheral blood lymphocytes induced by *P. acnes* pyridine extract residue in sarcoidosis patients and controls. (○—○) With prednisolone; (●—●) Without prednisolone; (×----×) Control.

tion index and the ACE activity ($r = 0.508$, $p < 0.1$) (Fig. 5). However, no significant correlation was noted between the stimulation index and the positive ^{67}Ga-scintigram.

Stimulation index of lymphocytes in peripheral blood induced by P. acnes. In 18 untreated sarcoidosis patients, the mean stimulation index was 0.81 at a concentration of *P. acnes* PER of 0.5 μg/ml, 0.88 at 1.0 μg/ml, 1.03 at 5.0 μg/ml, 1.06 at 10.0 μg/ml, 0.88 at 50 μg/ml, 0.70 at 100 μg/ml, and 0.65 at 500 μg/ml. The peak was at 10.0 μg/ml (Fig. 6), but this peak was too low to be regarded as a specific response to *P. acnes*. The mean stimulation index was 1.00 ± 0.43 in the controls and 1.05 ± 0.45 in the treated sarcoidosis patients at a concentration of 10.0 μg/ml. There were no significant differences between the two groups.

Discussion

Homma, J. Y. and his co-worker attempted to find a specific etiological bacteriological agent of sarcoidosis, and isolated anaerobic *Corynebacterium* from biopsy specimens (lymph nodes, pleura, parenchymal tissue, *etc.*) of patients with sarcoidosis at higher incidences and higher concentrations than in patients with no sarcoidosis. However, the sera of patients with sarcoidosis showed specific elevation in neither the *P. acnes* antibody titer nor precipitating antibody titer. In addition, there was no significant difference in the intensity of skin reactions to *P. acnes* between sarcoidosis and non-sarcoidosis groups. Although it is not entirely certain from their data that *P. acnes* plays a major role in the pathogenesis of this disease, it does seem that *P. acnes* is a virulent and pathogenic agent of sarcoidosis.

Lung biopsies of sarcoidosis patients revealed alveolitis accompanied by infiltrations of mononuclear phagocytes and lymphocytes in the alveolar structure and epithelioid cell granulomas. Alveolitis appears to represent a very early lesion, possibly the initial lesion, of pulmonary sarcoidosis, because it is observed in early sarcoid lesions and its incidence decreases with the progress of parenchymal fibrosis(4).

BAL fluid taken from patients with active sarcoidosis has been reported to show an increased number of activated T lymphocytes and helper T-cells, enhanced capacity to release interleukin-II from lymphocytes and

interleukin-1 from macrophages and increased expression of Ia-like antigen on macrophages (6-11). It is also known alveolar T lymphocytes of patients with active sarcoidosis aggressively incorporate tritiated thymidine and spontaneously release interleukin-II (12). These data indicate that the alveolar cell immune response is abnormally accelerated by the disease. Though it has been implicated that T lymphocytes are activated by some unidentified antigen in the lungs, the mechanism of increasing lymphocyte counts and activation of lymphocytes is not well understood.

The present study demonstrates that the amount of tritiated thymidine incorporated into alveolar lymphocytes of patients with active sarcoidosis significantly increased compared with that of lymphocytes of inactive patients and controls, following induction with pyridine extract residue containing the main cell wall components of *P. acnes*. Untreated sarcoidosis patients showed a dose-dependent response curve with a peak at a concentration of 5.0 μg/ml. This proliferative response of alveolar lymphocytes indicates a specific reaction to *P. acnes*. However, no specific response was observed with lymphocytes from healthy controls, lung cancer patients and sarcoidosis patients given corticosteroids. These findings seem to suggest that the alveolar lymphocytes of sarcoidosis patients are sensitized by *P. acnes*. *P. acnes* may reasonably be considered to be a resident of the alveolar bed because it is one of the indigenous bacteria found in the skin and intestine, but the mechanism of activation of alveolar T lymphocytes by *P. acnes* is unknown.

In untreated sarcoidosis patients, the stimulation index was increased in proportion to the activity of serum lysozyme and the percentage of lymphocytes in BAL fluid. In addition, there was a low correlation between the stimulation index and the serum ACE activity. Because of the elevation of the serum lysozyme activity, the serum ACE activity and the percentage of lymphocytes in BAL fluid seem to be good parameters indicative of disease activity (13−15). The stimulation index indicates the stage of disease progress, since it is significantly higher in the active stage than in the inactive stage, and significantly higher in sarcoidosis patients at radiographic stages I and II than in those at stage III or controls. Rosen, Y. reported that alveolitis was significantly more prevalent at stage I than at stage III, and that pulmonary fibrosis without alveolitis was seen more commonly than fibrosis with alveolitis (4). These observations and our data support the concept that the increased stimulation index represents the severity of alveolitis in sarcoidosis.

It has been confirmed that alveolar T lymphocytes are stimulated to synthesize protein by *P. acnes*. The stimulated lymphocytes release interleukin-II which stimulates a clonal increase in the number of responsive T lymphocytes (*i.e.*, helper T-cells and cytotoxic T-cells), and release a monocyte chemotactic factor that promotes the accumulation of monocytes in the alveolar structure. The local production of these lymphokines may mediate the accumulation of monocytes and affector lymphocytes in some alveolitic areas, and play a critical role in the initiation and/or maintenance of alveolitis in sarcoidosis patients. Therefore, monocytes which migrate are differentiated into macrophages and epitheloid cells by some lymphokines (*i.e.*, the macrophage migration inhibitory factor) to induce sarcoid granulomas.

Borna, B. P. has shown in his studies of beryllium-induced granulomas that granulomas occurred in a strain of animals whose T lymphocyte count was significantly elevated after beryllium exposure while no granulomas developed in a strain of animals whose T lymphocytes were not increased (16). These

observations confirmed that alveolitis represents a very early lesion of pulmonary granulomatosis. *P. acnes* is well-known to be a nonspecific activator of macrophages and monokines, which are released nonspecifically by alveolar macrophages and induced by *P. acnes* may enhance a proliferative response of alveolar T lymphocytes(17). However, this assumption may be wrong because the proliferation of T lymphocytes was promoted only in untreated patients, and not in treated patients or controls. The T-lymphocyte count also increases in patients with hypersensitive pneumonitis. Since comparative studies are necessary, such an investigation has been initiated by us.

Most patients with active sarcoidosis have peripheral blood lymphocytopenia, especially fewer T lymphocytes and helper T-cells, and abnormal *in vitro* proliferative response of lymphocytes to phytohemagglutinin(7, 18). However, the response curve of peripheral lymphocytes to *P. acnes* was not dose-dependent in patients with sarcoidosis and controls subjects. Furthermore, the stimulation indices were all below 1.0 in the patients, showing no difference depending on the concentrations of *P. acnes*. These observations suggest that the peripheral lymphocytes are not stimulated specifically by *P. acnes* in either sarcoidosis patients or controls.

In conclusion, alveolar lymphocytes in sarcoidosis have been found to be sensitized by *P. acnes* as demonstrated by increased incorporation of ^{3}H-thymidine by lymphocytes exposed to *P. acnes*. The stimulation index increased only in active sarcoidosis in proportion to the increased activity of serum lysozyme and the increased number of lymphocytes in BAL fluid. These observations suggested that the degree of alveolar lymphocyte proliferative response to *P. acnes* could be a good parameter of disease activity. Therefore, it is reasonable to hypothesize that alveolitis and infiltration of alveoli by lymphocytes activated by exposure to *P. acnes* are found at an early stage of sarcoidosis, followed by the developement of epitheloid cell granulomas at alveolitis sites, mediated by some mediators released from the alveolar lymphocytes.

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