

Analysis of Responses of Peripheral Blood Lymphocytes From Sarcoidosis Patients to Purified Protein Derivative

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Purpose: Negative skin test reaction against purified protein derivative (PPD) is one of the important diagnostic criteria for sarcoidosis. The purpose of this study was to investigate the relationship between negative skin test reaction against PPD and the responses of peripheral blood lymphocytes (PBLs) against PPD in sarcoidosis patients.

Methods: Sarcoidosis patients (n = 14) with ocular changes were selected for this study. As a control, blood was collected from volunteers without uveitis (n = 10). All subjects in both groups had a history of bacille de Calmette-Guerin vaccination, and 8 of the 14 patients underwent the PPD skin test. Peripheral blood lymphocytes were obtained from all patients and separated by gradient centrifugation. Peripheral blood lymphocytes were tested for their proliferative responses to PPD. To determine the frequency of PPD-specific precursor cells in blood, limiting dilution analysis was used. The frequency was calculated following the Poisson model. The culture supernatants were harvested 3 days after incubation and assayed for interferon (IFN)- γ production by ELISA.

Results: Four of the 8 patients showed a negative skin test reaction against PPD, while all the healthy volunteers had a positive reaction. Compared to PBLs from healthy volunteers, PBLs from sarcoidosis patients showed weaker responses to PPD. There was no clear difference between these two groups about frequency of precursor cells against PPD and production of IFN- γ .

Conclusions: Negative skin test for PPD in sarcoidosis patients does not seem to have any relationship to the low precursor frequency of PPD-specific cells in patient blood. **Jpn J Ophthalmol 2000;44:165-170** © 2000 Japanese Ophthalmological Society

Key Words: Frequency analysis, proliferation assay, purified protein derivative, sarcoidosis, tolerance.

Introduction

Sarcoidosis is a systemic granulomatous disease affecting various organs, such as eyes and lungs.^{1,2} This disease is thought to be immune-mediated, although target antigens or pathogens remain undefined.³ The major histological feature of affected tissues is noncaseating granuloma⁴ and it is quite similar to what is seen in tuberculosis. In addition, genome of atypical *mycobacterium* was detected from the inflamed tissue,^{5,6} thus suggesting the possi-

ble involvement of *mycobacterium*. Purified protein derivative (PPD) is a component of *Mycobacterium tuberculosis* and is known as a potent immunogen that inclines immune responses toward Th1 type immunity.⁷ Accordingly, infiltrated cells in the granuloma of sarcoidosis patients dominantly express Th1-type cytokines such as IL-12.^{8,9}

One important immunological finding often seen in sarcoidosis patients is hyposensitivity to PPD, and this finding is one of the important diagnostic criteria for sarcoidosis.¹⁰ Although this phenomenon is well-accepted among clinicians, the exact mechanism has not been fully elucidated.¹¹ As skin test reaction is considered to be a delayed type of hypersensitivity to PPD, there should be an adequate number of PPD-specific immunocompetent cells that can be-

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come effector cells at the site. Several hypothetical factors including anergy were considered as functional deficits of cells.¹⁰

Negative skin reaction might be due to an inadequate number of PPD-specific cells, but this has not been investigated. To study the relationship between the skin test for PPD and the responses of peripheral blood lymphocytes (PBLs) to PPD in sarcoidosis patients, frequency analysis for PPD-specific PBLs by the limiting dilution method and IFN- γ production by these PBLs, as well as proliferation assays, were performed.

Materials and Methods

Blood Donors

Fourteen sarcoidosis patients (3 men and 11 women, mean age: 59.1 ± 13.6 years) and 10 normal volunteers (9 men and 1 woman, mean age: 43.1 ± 20.5 years) gave informed consent to participate in this study, which adhered to the provisions of the Declaration of Helsinki. Sarcoidosis was diagnosed following the criteria for sarcoidosis set by the Ministry of Health and Welfare, Japan. For patients whose skin test data were not available, histologic examination records were confirmed for the diagnosis or three laboratory examination data, including high levels of serum angiotensin-converting enzyme were demonstrated. Sarcoidosis patients with ocular changes were selected for this study. Patients enrolled in this

study had not been receiving steroid therapy for at least 6 months. Controls for this study were normal volunteers without the ocular changes seen in sarcoidosis patients. All participants in both groups had a history of bacille de Calmette-Guérin (BCG) vaccination. The skin test against PPD was performed conventionally. Skin test reaction was divided into positive (diameter of redness: >10 mm), false-positive (5–9 mm), or negative (<4 mm). Heparinized blood was collected from all participants and PBLs were separated by gradient centrifugation (Lymphocyte Separation Medium; Organon Teknika, West Chester, PA, USA).

Proliferation Assay

Peripheral blood lymphocytes were cultured in 96-well flat-bottomed microplates at 2×10^5 cells per well, in 0.2 mL RPMI 1640 medium with HEPES, supplemented with 5% fetal calf serum with PPD at 0.001, 0.01, 0.1, 1 $\mu\text{g/mL}$, or 1 $\mu\text{g/mL}$ of phytohemagglutinin. After incubation for 100 hours, at 37°C, with 5% CO₂ and 100% humidity, cultures were pulsed for 16 hours with ³H-thymidine and harvested on a cell harvester. Data on proliferative responses were indicated as stimulation index (SI: ³H-thymidine incorporation of stimulated cells/that of unstimulated cells) and delta-count per minute (delta-cpm: ³H-thymidine incorporation of stimulated cells minus that of unstimulated cells).

Table 1. Patient Summary

| Case Number | Sex | Age | Skin Test | SI (0.1)* | SI (1)* | Delta-cpm (0.1)* | Delta-cpm (1)* | Frequency ($\times 10^{-5}$) | IFN- γ (pg/mL) |
|-------------|-----|------|-----------|-----------|---------|------------------|----------------|--------------------------------|-----------------------|
| 1 | M | 43 | FP | 4.3 | 10.1 | 12,292 | 13015 | NA | NA |
| 2 | F | 44 | NA | 3.1 | 2.3 | 967 | 2685 | 0.37 | 129 |
| 3 | F | 65 | FP | 14.5 | 15.3 | 1330 | 2755 | 4.03 | NA |
| 4 | F | 56 | P | 7.4 | 14.4 | 426 | 929 | NA | 0 |
| 5 | F | 70 | NA | 2 | 3.3 | 1077 | 1104 | NA | NA |
| 6 | M | 28 | N | 2.5 | 2.6 | 2181 | 3259 | 2.08 | 0 |
| 7 | F | 76 | N | 2.5 | 7.6 | 3771 | 4049 | NA | NA |
| 8 | F | 59 | NA | 2.4 | 2.4 | 1956 | 8705 | 1.26 | 0 |
| 9 | F | 65 | P | 12.9 | 14.3 | 19,873 | 24474 | 6.65 | 1080 |
| 10 | F | 63 | N | 4.3 | 5.1 | 8616 | 9558 | 4.83 | 133 |
| 11 | F | 65 | NA | 0.6 | 0.8 | 0 | 0 | 5.49 | 543 |
| 12 | F | 55 | N | 0.9 | 1.8 | 279 | 273 | NA | 71 |
| 13 | M | 79 | NA | 1.9 | 1.9 | 14,962 | 9440 | NA | NA |
| 14 | M | 59 | NA | 3.3 | 4.5 | 0 | 11371 | 2.45 | NA |
| Mean | | 59.1 | | 4.47 | 6.17 | 4838 | 6544 | 3.4 | 244.5 |
| SD | | 13.6 | | 4.26 | 5.23 | 6465 | 6784 | 2.2 | 382 |

SI: stimulation index, cpm: counts per minute, IFN: interferon, M: male, F: female, FP: false positive, NA: not available, P: positive, N: negative.

*Proliferative responses against purified protein derivative were evaluated by either SI or delta-cpm at two different concentrations (0.1 $\mu\text{g/mL}$ or 1 $\mu\text{g/mL}$).

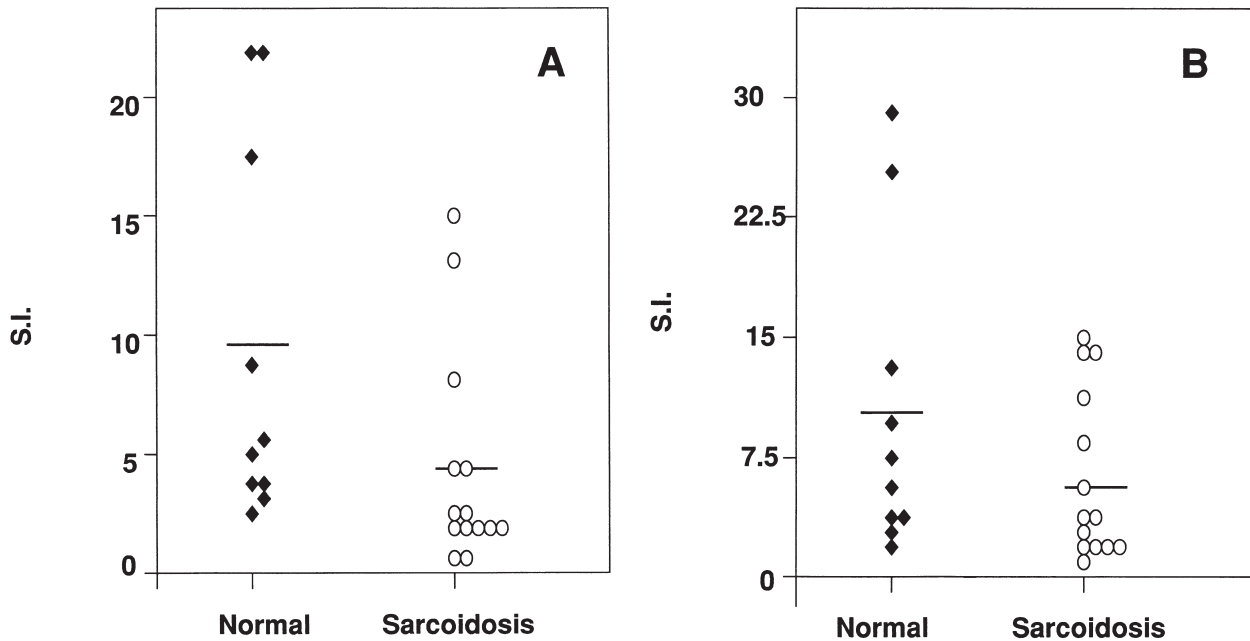


Figure 1. Proliferative responses of peripheral blood lymphocytes (PBLs) from either sarcoidosis patients or normal volunteers to purified protein derivative (PPD). PBLs were separated by gradient centrifugation and cultured with PPD at 0.1 µg/mL (A) or 1 µg/mL (B) for 5 days. Data are presented as stimulation indices (SI) and each dot indicates individual subject. Bar indicates mean SI of each group. Background of $^3\text{H-TdR}$ ranged from 97 to 19,624 in sarcoidosis patients and from 106 to 9324 in normal volunteers.

Frequency Analysis

Culturing by limiting dilution was used for this analysis. The culture method was basically the same as for proliferation assay, except for the number of cultured PBLs; 800, 1600, 3200, 6400, 12,800, 25,600, 51,200, 102,400 PBLs were cultured with or without PPD at 5 µg/mL as shown above. Average of cpm in each cell number without PPD was calculated and cpm of the well with PPD, which was more than average +3 SD, was regarded as a positive well. Then, positive wells were counted and the frequency estimate was calculated and fit to the Poisson model.¹²

Measurement of IFN- γ in Culture Supernatant

PBLs (1×10^6) were cultured in 96-well flat-bottomed microplates with PPD at 1 µg/mL following the method used in the proliferation assay. Culture supernatant was collected 3 days later and kept frozen until assay. To detect IFN- γ in the culture supernatants, ELISA (BioSource International, Camarillo, CA, USA) was performed strictly following the manufacturer's recommended procedure.

Statistical Analysis

Statistical analysis of proliferative responses, frequency analysis and IFN- γ production were per-

formed by Mann-Whitney *U*-test comparing the data from sarcoidosis patients and normal volunteers. $P < .05$ was regarded as statistically significant.

Results

PPD Skin Tests in Sarcoidosis Patients

Skin test data were available for 8 of the 14 patients who were enrolled in this study. Two of the 8 patients demonstrated positive skin test reaction against PPD, while 4 patients were negative and 2 patients were false-positive (Table 1). In contrast, all healthy donors exhibited positive skin reaction against PPD.

PBLs from Sarcoidosis Patients Showed Weaker Proliferative Responses to PPD

To investigate the relationship between the responses of PBLs and the skin test, cellular proliferative assays were employed. Data are presented as SI (Figures 1A,B) and delta-cpm (Figures 2A,B). PBLs from healthy volunteers responded to PPD vigorously at both 0.1 µg/mL (Figure 1A, average \pm SD = 9.15 \pm 8.09; Figure 2A, average \pm SD = 14083 \pm 11022) and 1 µg/mL (Figure 1B, average \pm SD = 10.06 \pm 9.13; Figure 2B, average \pm SD = 14081 \pm 11022). Compared to PBLs from normal volunteers, PBLs from

sarcoidosis patients showed weaker responses to PPD at both concentrations (Figure 1A, average \pm SD = 4.47 ± 4.26 ; Figure 2A, average \pm SD = 4838 ± 6465 ; Figure 1B, average \pm SD = 6.17 ± 5.23 ; Figure 2B, average \pm SD = 6544 ± 6784). The difference is more apparent at 0.1 g/mL. Data represented by delta-cpm between normal and sarcoidosis patients were statistically different at $P = .011$ (0.1 μ g/mL) or $.009$ (1 μ g/mL). Statistical significance in the data represented by the SI was demonstrated only at 0.1 μ g/mL ($P = .047$ at 0.1 μ g/mL, $P = .188$ at 1 μ g/mL). The correlation between proliferative responses and skin test data is summarized in Table 1.

Frequency Analysis of PPD-Specific PBLs from Either Healthy Volunteers or Sarcoidosis Patients

Differences in proliferative responses between sarcoidosis patients and healthy volunteers could be attributed to the different numbers of PPD-specific precursor cells in peripheral blood. To investigate the difference in numbers of precursor cells specific to PPD, frequency analysis was performed. The average of precursor frequency in sarcoidosis patients was 3.40×10^{-5} (\pm SD = $\pm 2.20 \times 10^{-5}$) (Figure 3, Table 1), while that in healthy volunteers was 5.43×10^{-5} (\pm SD = $\pm 6.35 \times 10^{-5}$) (Figure 3). There was no statistically significant difference between the two groups ($P = .817$).

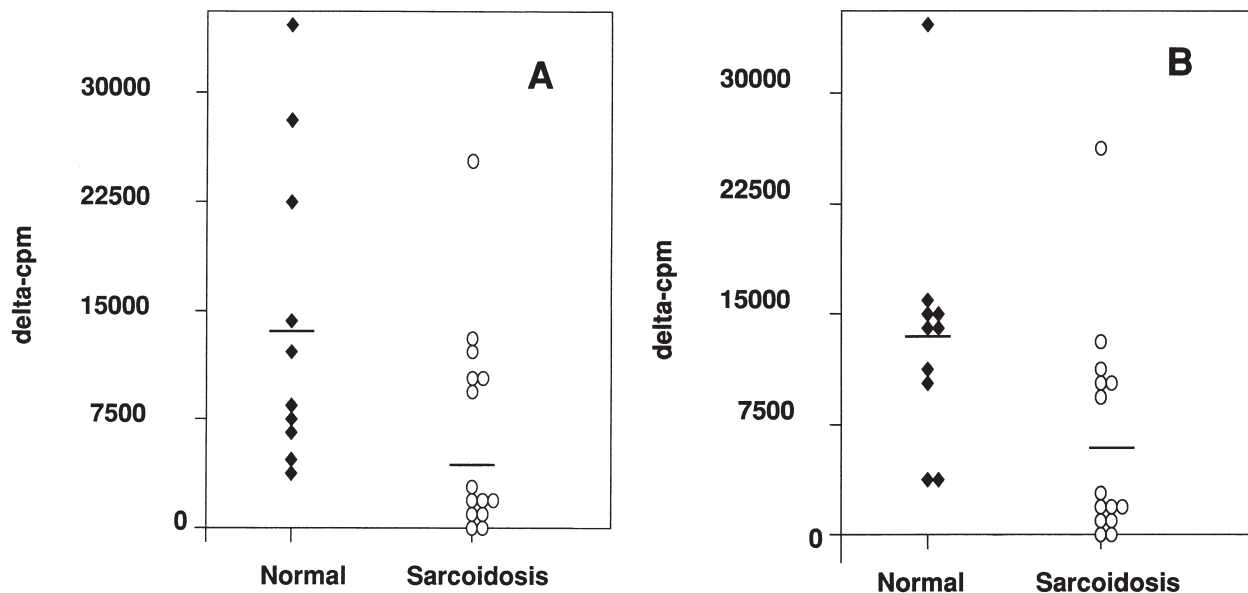


Figure 2. Proliferative responses of peripheral blood lymphocytes (PBLs) either from sarcoidosis patients or normal volunteers to purified protein derivative (PPD). PBLs were separated and cultured with PPD at 0.1 μ g/mL (A) or 1 μ g/mL (B) for 5 days. Data are presented as delta-cpm (count per minute) and each dot indicates individual subject. Bar indicates mean delta-cpm of each group. Background of 3 H-TdR ranged from 97 to 19,624 in sarcoidosis patients and from 106 to 9324 in normal volunteers.

IFN- γ Production in the Culture Supernatant of PPD-Stimulated PBLs

Another hypothetical explanation for different proliferative responses of PBLs to PPD is the different immunological properties of PPD-specific cells in spite of the same number of precursors. To investigate this theory, we tested the production of IFN- γ in culture supernatant produced by PBLs stimulated with PPD in vitro. The average of IFN- γ produced in sarcoidosis patients was 244.5 pg/mL (\pm SD = ± 382) (Figure 4, Table 1), while that in healthy volunteers was 145 pg/mL (\pm SD = ± 150) (Figure 4). There was no statistically significant difference between these two groups ($P = .734$).

Discussion

Even though 4 of the 8 sarcoidosis patients examined exhibited negative skin reaction and the number of subjects is relatively smaller than in previous reports,^{13,14} our data demonstrated that PBLs from sarcoidosis patients tend to show weaker responses to PPD than PBLs from healthy donors. Our data, as well as previous reports,^{13,14} could be interpreted to indicate that immunotolerance to PPD exists in sarcoidosis patients. Although this phenomenon is well-accepted and anergy in cellular immunity was considered to be the main reason for this tolerance,¹⁵ no

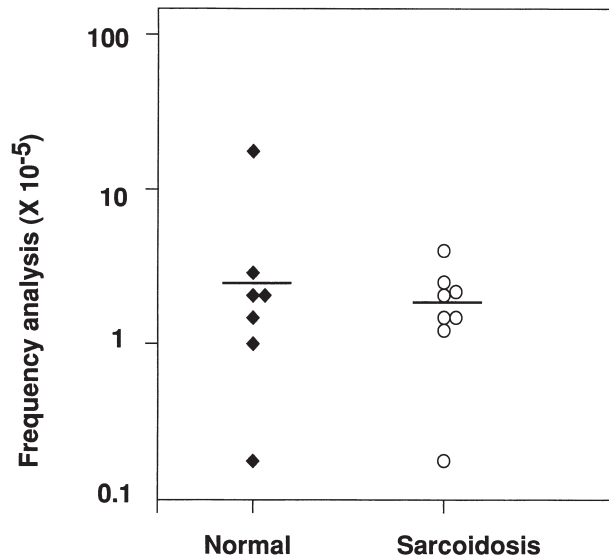


Figure 3. Comparison of frequency of purified protein derivative (PPD)-specific peripheral blood lymphocytes (PBLs) between sarcoidosis patients and normal volunteers. Different numbers of PBLs were cultured with PPD at 5 $\mu\text{g}/\text{mL}$ for 5 days and uptake of ^3H -TdR was measured. Positive wells were counted and frequency estimate was calculated and fit to Poisson model. Data are recorded as PPD-specific frequency per 1,000 PBLs. Bar indicates mean value of frequency.

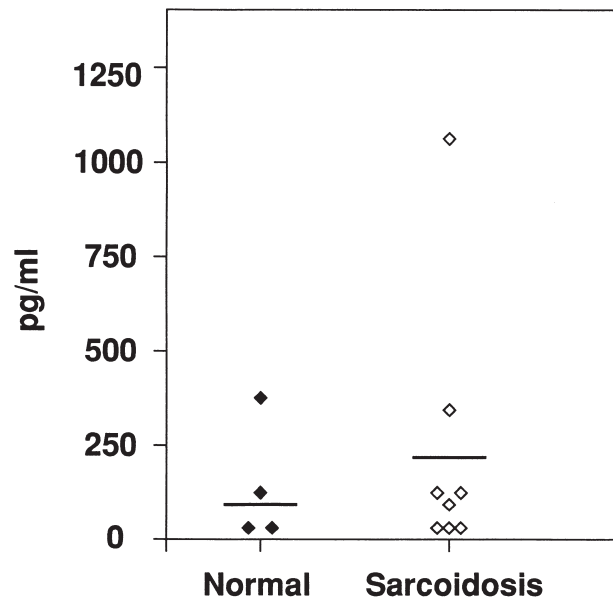


Figure 4. Comparison of interferon (IFN)- γ production in peripheral blood lymphocytes (PBLs) stimulated with purified protein derivative (PPD) in sarcoidosis patients and normal volunteers. PBLs were stimulated with PPD for 3 days and supernatants were harvested for ELISA. Data are represented as pg/mL. Bar indicates mean value of IFN- γ .

direct evidence emerged to support the case for anergy.¹⁶ This led us to investigate the mechanism for hyposensitivity to PPD in sarcoidosis patients.

In general, the mechanisms for induction of tolerance could be divided into deletion, anergy, and active suppression.¹⁷ First, we tested the possibility of clonal deletion of PPD-specific PBLs by frequency analysis. The data suggest that clonal deletion was not involved in this hyposensitivity to PPD. It should be interpreted that there are cells specific to PPD in peripheral blood from sarcoidosis patients, although these cells were not able to respond well to PPD compared to those from healthy subjects. To the best of our knowledge, this is the first report which seems to rule out the involvement of clonal deletion in hyposensitivity to PPD in sarcoidosis patients.

Th1 type cytokines rather than Th2 type cytokines are detected in the inflamed tissues from sarcoidosis patients,^{8,9} suggesting the dominant role of Th1 type cytokines in the development of this disease. It is well-known that cytokines from Th1 type T cells tend to inhibit Th2 type T cells,^{18,19} and those from Th2 type T cells inhibit Th1 type T cells.^{18,19} Th1 type T cells are generally effector cells with delayed sensitivity,^{18,19} thus inducing granuloma formation. Therefore, it

could be considered that more Th1 type cytokine, such as IFN- γ , in sarcoidosis patients may be produced by PBLs stimulated with PPD. On the other hand, IFN- γ has cytotoxic ability,²⁰ and excessive production of this cytokine may lead to inhibition of cellular proliferation. To investigate the possible effect of excessive IFN- γ production on the hyposensitivity of PBLs to PPD in sarcoidosis patients, we tested production of IFN- γ by these cells after stimulation with PPD. No differences in IFN- γ production were observed between these two groups, indicating that IFN- γ does not seem to be involved in the inhibition of proliferation of PBLs by in vitro stimulation with PPD. Other cytokines, such as IL-4 and IL-12, should be measured to demonstrate whether cytokine imbalance is involved in this hyposensitivity.

As shown in all figures, there are variations in the data within each group. To compare the immune responses and skin test individually, individual data were summarized in Table 1. Although skin test data were available in only 8 patients, patients with negative skin test reaction tend to show weaker proliferative responses than patients with positive skin test reaction. The data further indicate the relationship between skin test and proliferative responses. There

seem to be no apparent differences in precursor frequency, thus indicating that clonal deletion is not involved in the negative skin test in sarcoidosis patients.

Another possible mechanism for this hyposensitivity is that lymphocytes specific for PPD may accumulate in the inflammatory sites such as hilar lymph nodes,²¹ where PPD-like presumed target antigens exist; therefore, less proliferative response would be detected in PBLs from sarcoidosis patients. To investigate this possibility, inflamed organs should be harvested and infiltrated lymphocytes should be collected for proliferation assay. Unfortunately, in this study, we were not able to obtain lymphocytes from lymph nodes for this assay.

Other antigens, which are not related to products of *Mycobacterium*, should be investigated to demonstrate that this hyposensitivity is antigen-specific. Lecossier et al²² demonstrated that sarcoidosis patients have a lack of responsiveness to *Bordetella pertussis*, thus concluding that nonspecific anergy is the suspected mechanism of hyposensitivity to PPD. It is well-known that anergy can be broken down by the addition of IL-2.^{23,24} The role of anergy in the hyposensitivity of PBLs from sarcoidosis patients is now currently under investigation by the addition of IL-2 to a culture in vitro. This experiment will help elucidate the role of anergy in the hyposensitivity of sarcoidosis patients to PPD.

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