Cooperation between Accessory Cells and
T Lymphocytes in Patients with
Tuberculous Pleurisy*

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We studied interleukin 1 (IL-1) activity of pleural fluid macrophages and peripheral blood monocytes obtained from ten patients with tuberculous pleurisy and ten patients with malignant pleurisy, using purified protein derivative (PPD) as a stimulating agent. Tuberculous pleural fluid macrophages and peripheral blood monocytes tended to produce higher IL-1 activity than malignant pleural fluid macrophages and blood monocytes and showed significantly more IL-1 activity than healthy control monocytes. However, no significant difference in IL-1 activity was observed between tuberculous pleural macrophages and blood monocytes. With the cooperation of these accessory cells, pleural fluid T lymphocytes in patients with tuberculous pleurisy showed a significant level of interleukin 2 (IL-2) activity in the presence of PPD. Tuberculous pleural fluid macrophages promoted greater IL-2 production than blood monocytes from either tuberculous pleural fluid or blood T lymphocytes despite relative equivalence in measured IL-1 production. Combination of tuberculous pleural fluid macrophages and pleural fluid T lymphocytes was the most effective for increasing IL-2 activity when compared with other combinations. These results suggest that tuberculous pleural fluid macrophages and T lymphocytes may contribute to the immunopathogenesis of tuberculosis at a local site of disease.

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FCS = fetal calf serum; IL-1 = interleukin 1; IL-2 = interleukin 2; LPS = lipopolysaccharide; PBS = phosphate-buffered saline solution; PPD = purified protein derivative

The importance of macrophages in the defense mechanism of the lung has been demonstrated by many investigators; alveolar macrophages especially have been well reviewed. Their secretory and regulatory functions in health and disease have been frequently studied and many conflicting results have been reported. On the other hand, pleural fluid macrophages have been least reviewed, and the role and function of these cells are mostly unknown, partly because they are not always readily accessible.1

In the study of tuberculous pleural effusions, a number of interesting articles have reported preferential sequestration of antigen-reactive lymphocytes in the pleural spaces and the higher proliferative response to purified protein derivative (PPD) of pleural fluid T lymphocytes rather than circulating blood T lymphocytes.2-4 However, little is known concerning the immunoregulatory function of tuberculous pleural fluid macrophages. Alveolar macrophages of healthy subjects were reported to be as adept as circulating blood monocytes in providing accessory function for responses to antigen at low ratios.5 However, alveolar macrophages generally secrete less interleukin 1 (IL-1) in response to lipopolysaccharide (LPS) than blood monocytes,6 and IL-1 production by pleural macrophages is unknown. Meanwhile, circulating blood monocytes act as antigen-specific suppressor cells patients with active pulmonary tuberculosis.7,8 Therefore, it is of great interest to elucidate the immunoregulatory capacity and secretory functions of tuberculous pleural fluid macrophages.

IL-1 is a soluble factor secreted by stimulated mononuclear phagocytes that is thought to have immunoregulatory effects and multiple biologic activities. In vitro, IL-1 has been shown to stimulate T lymphocytes and enhance interleukin 2 (IL-2) production.9-12 LPS is a standard stimulus for production of IL-1, and PPD was recently demonstrated to stimulate monocyte release of IL-1.13 Therefore, we adopted PPD as a more disease-specific stimulus in patients with tuberculosis. Defective IL-2 production and responsiveness in human pulmonary tuberculosis have also been reported.14

The purpose of this study is as follows: First, we attempted to compare IL-1 activity of tuberculous pleural fluid macrophages with that of blood monocytes of the same donor in response to PPD. Second, we sought to compare the IL-2 activity of tuberculous pleural fluid T lymphocytes in response to PPD with that in blood T lymphocytes of the same donor. In addition, the capacity of each of these accessory cells to enhance autologous T-lymphocyte responses in IL-2 activity was estimated.

METHODS

Subjects

Ten patients from 16 to 77 years of age with newly diagnosed
adenocarcinoma, and ten patients from 55 to 77 years of age with newly diagnosed malignant pleurisy (eight patients with adenocarcinoma, one patient with malignant lymphoma, and one patient with mesothelioma) were studied before initiation of therapy. No patients with tuberculous pleurisy had miliary tuberculosis or serious underlying diseases. Diagnosis was made on the basis of positive pleural biopsy specimen or positive bacteriologic findings. Ten control subjects with positive tuberculin reaction were selected from laboratory and hospital staff. The control population was age matched to the patient population as closely as possible. Incubation of bacillus Calmette-Guérin vaccination in infancy is obligatory in Japan, and the incidence of tuberculosis in Japan has been rather high, so tuberculin-negative healthy control subjects could not be included in the present study.

Cell Preparations

Peripheral blood and pleural fluid samples from the same donor were collected on the same day, after obtaining the informed consent of all subjects. Pleural fluid was obtained by intercostal needle puncture and heparinized. Heparinized whole blood, 20 to 50 ml, was diluted 1:1 with phosphate-buffered saline solution (PBS). Pleural fluid or peripheral blood was layered on a Ficoll-Paque gradient (Pharmacia Fine Chemicals, Uppsala, Sweden). The preparation was centrifuged at 400 g for 30 minutes at room temperature. The band of mononuclear cells at the interface was removed from the gradient and the cells were washed twice with PBS.

To separate macrophages or monocytes from lymphocytes, mononuclear cells were plated onto T-75 plastic flasks (Corning Glass Works, Corning, NY) that were pretreated with fetal calf serum (FCS). Flasks were incubated at 37°C in 5 percent CO2-humidified air for one hour. Nonadherent cells were removed by repeated washing with RPMI-1640 medium (Nissui Pharmaceutical Co, Tokyo, Japan). Adherent cells were covered with 0.02 percent cold edetic acid plus 5 percent FCS and incubated at 4°C for 30 minutes. The cells were resuspended by mechanical agitation and washed three times in PBS. This technique yielded more than 90 percent nonspecific esterase positive cells and more than 95 percent viable cells by trypan blue dye exclusion.

T lymphocytes were identified by their ability to form rosettes with sheep red blood cells (SRBC) [7, 8]. Nonadherent cells were mixed with SRBC in FCS at a 1:20 ratio and incubated at 37°C for 15 minutes. The cells were then centrifuged at 170 g for 5 minutes before they were incubated at 4°C for 60 minutes. T lymphocytes that formed rosettes with SRBC were selectively isolated from mixed cell suspension by separating the rosettes by equilibrium density gradient centrifugation. The rosetting cells together with the SRBC were lysed with tris-NH4Cl solution. T lymphocytes thus obtained were further purified by depletion of OKM1-positive cells using the murine monoclonal antibody (Ortho, Raritan, NJ) and Low-Tox-H rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada). They were incubated (1 × 10^7 ml at 4°C) with 5 μl of OKM1 monoclonal antibody for 30 minutes, washed, and incubated at 37°C with rabbit complement for 45 minutes. The resulting cell population consisted of more than 99 percent lymphocytes (less than 1 percent nonspecific esterase positive).

Assay for Endotoxin

The PPD preparation was diluted in sterile pyrogen-free water. Sample of 0.2 ml was incubated with limulus lysate and chromogenic substrate for 30 minutes at 37°C. Endotoxin involved was measured by test kit (Toxicolor, Seikagaku Kogyo, Tokyo, Japan). The lower limit of sensitivity of this assay was 1 pg/ml.

Assay for IL-1 Activity

Pleural macrophages and blood monocytes were cultured in 24-well plates (Corning Glass Works, Corning, NY) at 5 × 10^5 cells/ml (1 ml/well) in RPMI-1640 medium containing 5 percent heat-inactivated FCS supplemented with 100 U/ml penicillin, 60 μg/ml kanamycin, and 2 mM L-glutamine. Cells were cultured alone or in the presence of 50 μg/L PPD (Mitsui Pharmaceutical Co, Tokyo, Japan). After 24 hours of incubation at 37°C in 5 percent CO2-humidified air, culture supernatants were separated from cells by centrifugation at 500 g for 10 minutes and stored at −20°C until assay. These culture intervals and conditions were adequate for optimal IL-1 activity in the supernatants. An IL-1 standard was prepared from normal peripheral blood adherent cells stimulated with LPS (10 μg/L, O127:B8, Difco, Detroit, Mich).

IL-1 activity of the supernatants was quantitated using the standard mouse thymocyte assay. Briefly, thymocytes were obtained from 4- to 5-week-old female C3H/He mice (Institute for Laboratory Animal Research of Nagoya University School of Medicine) and suspended in RPMI-1640 medium with 50 μg/ml gentamicin, 5 percent heat-inactivated FCS, and 2 × 10^−3 M 2-mercaptoethanol. These thymocytes were cultured with dilutions of the standard IL-1 sample or test supernatants at 1.5 × 10^4 cells/well in flat-bottomed microtiter plates (Corning Glass Works, Corning, NY) and just before plating, a suboptimal dose (2 mg/L) of phytohemagglutinin-P (PHA) (Difco Labs., Detroit, Michigan) was added to the thymocyte suspension. Each dilution was assayed in triplicate. Culture plates were incubated at 37°C in 5 percent CO2-humidified air for 48 hours, pulsed with 0.5 μCi of methyl-3H-thymidine (New England Nuclear, Boston, Mass) per well, and incubated for an additional 24 hours before harvesting onto glass fiber filter disks using a cell harvester. The 3H-thymidine content was assayed by liquid scintillation counter. IL-1 activity was quantitated using probit analysis.

PPD-Induced Blastogenesis

Highly purified blood and pleural fluid T lymphocytes were cultured in the presence of PPD (2.5 μg/L) in 96-well plates at 1 × 10^6/ml (0.2 μl/well), respectively, with or without 10 percent blood monocytes or 10 percent pleural fluid macrophages. After four days of incubation, culture cells were pulsed with 0.5 μCi of methyl-3H-thymidine for 12 hours and harvested, and the radioactivities were assayed by liquid scintillation counter.

Assay for IL-2 Activity

Highly purified blood and pleural fluid T lymphocytes were cultured, respectively, with autologous peripheral blood monocytes or pleural fluid macrophages in the presence of PPD (2.5 μg/L) at 37°C in 5 percent CO2-humidified air. After 24 hours of incubation, culture supernatants were centrifuged at 500 g for 10 minutes and stored at −20°C. These culture intervals and conditions were adequate for IL-2 activity. Human rIL-2 (Shionogi Pharmaceutical Co, Ltd, Osaka, Japan) was used as the standard IL-2 sample. Cell-free supernatants were assayed for IL-2 activity using the IL-2 dependent cell line HT-2. Serial dilutions of supernatants were made in flat-bottomed micro wells and 4 × 10^4 HT-2 cells were added to each well. Methyl-3H-thymidine was added after 24 hours of incubation and cultures were harvested 16 hours later. IL-2 activity was quantitated using probit analysis.

Statistical Analysis

Statistical significance was determined by analysis of variance, t-test, or regression analysis. P value less than 5 percent was considered significant.

Results

PPD-Induced IL-1 Activity

As shown in Figure 1, blood monocytes or pleural fluid macrophages acquired from patients with tuber-
PPD-induced interleukin 1 activities of pleural fluid macrophages (Me) and blood monocytes in patients with tuberculous pleurisy and malignant pleurisy. IL-1 activities of blood monocytes from PPD-positive control subjects were also studied. Pleural fluid macrophages and monocytes in patients with tuberculous pleurisy showed significantly more IL-1 activity than normal monocytes (p<0.05) and IL-1 activity tended to be higher than in cells of patients with malignant pleurisy. There was no significant difference between the IL-1 activities of pleural fluid macrophages and monocytes in patients with tuberculous pleurisy.

Tuberculous pleurisy showed significantly more IL-1 activity than blood monocytes from normal subjects when stimulated with PPD (p<0.05). Pleural fluid macrophages and blood monocytes were studied in eight patients with malignant pleurisy. Tuberculous pleural fluid macrophages and peripheral blood monocytes tended to produce more IL-1 activity than cells from patients with malignant pleurisy when stimulated with PPD (0.05<p<0.1). No significant correlation of PPD-induced IL-1 activity was found between autologous pleural fluid macrophages and blood monocytes in patients with tuberculous pleurisy (r = 0.18). The PPD preparation contained 0.0045 percent LPS by weight. Under conditions of cell culture, therefore, PPD contained 2.25 pg/ml LPS.

Table 1 — PPD-Induced Blastogenesis and IL-2 Activity with or without Accessory Cells in Tuberculosis*

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<td>NT</td>
</tr>
<tr>
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<td>19,102</td>
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*NT = not tested.
**PPD-Induced Blastogenesis**

We observed PPD-induced lymphocyte proliferation (Table 1). Both pleural fluid macrophages and blood monocytes markedly promoted lymphocyte proliferation. The accessory cell function of pleural fluid macrophages was slightly higher than that of blood monocytes. Pleural fluid T lymphocytes responded far more to PPD stimulation with the addition of accessory cells when compared with blood T lymphocytes.

**PPD-Induced IL-2 Activity**

Purified tuberculous peripheral blood and pleural fluid T lymphocytes were reconstituted with autologous purified blood monocytes and pleural fluid macrophages, respectively. The supportive effects of tuberculous blood monocytes and pleural fluid macrophages involved with PPD-induced IL-2 activity were compared in this attempt, in which both types of accessory cells, in various amounts, were added to autologous blood and pleural fluid T lymphocytes (Fig 2). Supernatants of reconstituted cells, which were cocultured with PPD, showed an increase of IL-2 activity in terms of the number of accessory cells. The combination of pleural fluid T lymphocytes and pleural fluid macrophages appeared to be the most effective. Conversely, the supernatants acquired from combination of blood T lymphocytes and blood monocytes showed the lowest IL-2 activity. The other two combinations ranked in between.

In Figure 3, PPD-induced IL-2 activities of T lymphocytes with autologous accessory cells were compared in ten patients with tuberculous pleurisy. As shown in Figure 3A, the combination of tuberculous pleural fluid T lymphocytes with 10 percent pleural fluid macrophages yielded more PPD-induced IL-2 activity than the combination of autologous blood T lymphocytes with 10 percent blood monocytes (p<0.01). However, the latter combination was not significantly effective when compared with the same cell combination from healthy tuberculin-positive subjects (Fig 3A). Combination of tuberculous pleural fluid T lymphocytes and 10 percent autologous pleural fluid macrophages showed significantly increased PPD-induced IL-2 activity when compared with combination of autologous pleural fluid T lymphocytes and blood monocytes (p<0.01) (Fig 3B). Even when tuberculous pleural fluid T lymphocytes were replaced by autologous blood T lymphocytes, the addition of
autologous pleural fluid macrophages was more effective than with autologous blood monocytes (p<0.05) (Fig 3C). These observations suggest that, when added to autologous T lymphocytes, tuberculous pleural fluid macrophages act more effectively as accessory cells in specific antigen PPD-induced IL-2 activity than blood monocytes. Furthermore, with the cooperation of identical accessory cells, the responses of the two kinds of T lymphocytes in PPD-induced IL-2 activity (Fig 3D, 3E) were remarkably different. Tuberculous pleural fluid T lymphocytes released more IL-2 activity than autologous blood T lymphocytes (p<0.01).

As shown in Figure 4, PPD-induced IL-2 activities from combination of T lymphocytes and autologous accessory cells obtained from ten patients with tuberculous pleurisy and ten patients with malignant pleurisy were compared. There were significant differences between IL-2 activities of pleural fluid combination (p<0.02) and between IL-2 activities of peripheral blood combinations (p<0.05).

IL-2 activities stimulated by PPD with the cell combination of autologous pleural T lymphocytes plus 10 percent pleural fluid macrophages and with that of autologous blood T lymphocytes plus 10 percent monocytes were compared in ten patients with tuberculous pleurisy. There was a significant correlation between both IL-2 titers (p<0.02), although IL-2 activity from the former combination was far higher than that of the latter (Fig 5).

DISCUSSION

We undertook these studies to investigate PPD-induced IL-1 activity of tuberculous pleural fluid macrophages and blood monocytes, and to elucidate the influence of these accessory cells on PPD-induced IL-2 activity of pleural fluid T lymphocytes and blood T lymphocytes. LPS is commonly used for stimulation of IL-1 release in vitro, but it is not a physiologic stimulus for IL-1 release in vivo in tuberculosis. Therefore, we used PPD for IL-1 and IL-2 release in the present study. The effect of PPD on IL-1 activity did not appear to be due to its contamination by LPS, because the PPD used contained only 0.0045 percent LPS by weight. As shown in the present study, tuberculous pleural fluid macrophages promoted PPD-induced IL-2 activity of autologous T lymphocytes more effectively than autologous blood monocytes, while there was no significant difference in the IL-1 activity of macrophages and monocytes of patients. Therefore, factors other than IL-1 may also contribute to IL-2 production in T lymphocytes. Suppressive adherent cells may be present, for example, and suppressive factors may be released in greater amounts by monocytes relative to macrophages.8,25 As expected, PPD-induced IL-1 and IL-2 activities in patients with tuberculous pleurisy tended to be higher, if not significantly higher, than in patients with malignant pleurisy.

Some current evidence suggests that IL-1 acts as an amplifying factor rather than an essential signal for IL-2 production.26,27 Recently, Fujiwara and colleagues26 demonstrated that monocytes from patients with tuberculosis were activated to produce higher IL-1 levels than those from healthy subjects when stimulated with LPS or PPD. This finding shows partial agreement with ours. Circulating blood monocytes are believed to act as antigen-specific suppressor cells in patients with active pulmonary tuberculosis.7,8 In the present investigation of PPD-induced IL-2...
activity, tuberculous pleural fluid macrophages and blood monocytes of the same patient served to activate autologous T lymphocytes. Interestingly, pleural fluid macrophages acted more effectively than blood monocytes. A much earlier study indicated that, under high mycobacterial load, adherent cells were activated to release suppressor factors that caused lymphocytes to become suppressor cells. Usually, tuberculous pleurisy is recognized at an early and as yet undestructive stage of lung tuberculosis. This may be why no suppressive effects of adherent cells were observed in our series.

Alveolar macrophages have been frequently reviewed and well studied for their accessory effects. Previous studies demonstrated that alveolar macrophages are relatively poor accessory cells for the stimulation of antigen-induced lymphocyte proliferation compared with blood monocytes. Alveolar macrophages are fully capable of accessory activity when they exist at low ratios to lymphocytes in vitro. At high ratios, suppression by alveolar macrophages predominates, whereas in blood monocytes the reverse is true. Our present studies suggested that tuberculous pleural fluid macrophages act as accessory cells to equal or surpass blood monocytes at least in regard to IL-2 activity of T lymphocytes. Moreover, no suppressive effect of pleural fluid macrophages on IL-2 activity was observed even when the macrophages were added in high ratios to T lymphocytes. Thus, there might be some difference in the suppressive effect between the types of macrophages.

It is well known that T lymphocytes play a major role in the defense of intracellular organisms. Both the proportion and the absolute number of T lymphocytes in tuberculous pleural effusion were reported to be significantly higher than in peripheral blood, and specific antigen PPD-reactive lymphocytes preferentially sequestrated in the pleural space. When T lymphocytes in tuberculous pleural effusion were cocultured with PPD, they showed a higher proliferative response to PPD and produced far more immune interferons than autologous blood lymphocytes. Sequestration of PPD-reactive T lymphocytes in the pleural space in patients with tuberculous pleurisy seems to be critical for the defense of lungs against invading acid-fast bacilli. In these cases, pleural fluid macrophages probably act more effectively as accessory cells than autologous blood monocytes and contribute to active local cellular immunity at the morbid site through the stimulation of PPD-reactive T lymphocytes.

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