

In Vitro Tuberculin Reactivity of Lymphocytes from Patients with Tuberculous Pleurisy

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Mononuclear cells in pleural fluid from patients with tuberculous pleurisy were predominantly T cells. Responsiveness of pleural fluid T cells to purified protein derivative of tuberculin were studied by the assay of cell proliferation and production of lymphocyte mitogenic factor by the stimulation with purified protein derivative. Peripheral blood lymphocytes were also studied from patients and tuberculin-positive healthy controls. The order of responsiveness was as follows: pleural fluid lymphocytes > peripheral blood lymphocytes of patients without effusion = peripheral blood lymphocytes of healthy controls > peripheral blood lymphocytes of patients with effusion. The poor response of peripheral blood lymphocytes from pleurisy patients were recovered by the elimination of adherent cells in peripheral blood lymphocytes to the level of the response of peripheral blood lymphocytes from healthy controls. T cells purified from pleural fluid mononuclear cells responded more than those from peripheral blood. These results suggested that in the pleurisy patients purified protein derivative-reactive T cells in peripheral blood did not decrease in activity, but were depressed by suppressor cells, and further suggested that highly purified protein derivative-reactive T cells were accumulated in the pleural fluid.

Tuberculous pleurisy with effusion is one of the diseases in which cell-mediated immunity might play an important role in pathogenesis. Although there are many immunological investigations from various points of view on pulmonary tuberculosis without effusion (1, 8, 12, 17, 18, 21, 22), there are few investigations, especially at the cellular level, on tuberculous pleurisy (6). Tuberculous pleural fluids contain lymphocytes, most of which belong to T cells (15, 16), in high concentration. In this regard it is intriguing to investigate and clarify the immunological characteristics of lymphocytes in pleural fluid.

In the present study, we examined the immunological functions of lymphocytes from patients with tuberculous pleurisy and pulmonary tuberculosis patients without pleurisy by using two in vitro assay systems: purified protein derivative of tuberculin (PPD)-induced proliferative response and PPD-induced production of lymphocyte mitogenic factor (LMF), both of which are reported to be associated with T-cell functions (4, 7, 9, 14).

MATERIALS AND METHODS

Subjects. Twenty-seven patients (27 to 69 years old, mean 42 years) with tuberculous pleurisy with effusion and 30 patients (18 to 64 years old, mean 37 years) with active pulmonary tuberculosis without effusion hospitalized at Osaka Prefectural Habikino Hospital were

examined before the initiation of chemotherapy. All patients with tuberculous pleurisy were newly detected and had tuberculous lesions in their lungs. Diagnosis was based on the histological findings of biopsied specimen of pleura. All patients with pulmonary tuberculosis were also newly detected and diagnosed by the demonstration of acid-fast bacilli in sputa. No patients had miliary tuberculosis or underlying diseases. All patients were positive for the tuberculin skin test with 5 tuberculin units (TU) of PPD, except for two patients with pleurisy. Forty-one healthy hospital employees and laboratory workers (19 to 49 years old, mean 29 years) that were all positive for the tuberculin skin test served as control subjects. The patients and controls were not immunized with BCG at least within 1 year before study. Age-matched controls were not necessary for the comparison with the patients because the patients over 50 years old had in vitro PPD responses like those of the younger patients; age did not appear to affect the responses.

Isolation and characterization of MNC. Mononuclear cells (MNC) in peripheral blood were isolated from heparinized peripheral blood by Ficoll-Hypaque (specific gravity, 1.077) density centrifugation (3). Pleural fluid MNC were obtained from heparinized pleural fluid by the same method as for peripheral blood. Blood and pleural fluid samples were collected on the same day. Rosette-forming cells were tested for their ability to form rosettes with sheep erythrocytes (SRBC) by the method of Jondal et al. (11), except for the initial incubation period of 20 min of MNC with SRBC at 37°C. To stabilize the rosettes, MNC and SRBC were suspended in 100% fetal bovine serum (Microbiological Associates, Walkersville, Md.).

More than 200 MNC were counted; samples with MNC binding more than three SRBC were considered positive. Surface immunoglobulin-bearing cells were detected by staining with fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin serum (polyvalent anti-immunoglobulin G, M, and A; Behringwerke AG, Marburg, Germany). MNC (5×10^5) in 1 ml of RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan) were incubated at 37°C, in 5% CO₂ in air. After 24 h, they were washed twice and then incubated with fluorescein isothiocyanate-conjugated anti-human immunoglobulin serum for 60 min at 4°C, followed by three washings in phosphate-buffered saline. The pellet was resuspended in one drop of glycerol-phosphate-buffered saline (1:1). Over 200 MNC were counted by using an Olympus model BH-RFL fluorescence microscope, and cells displaying more than three sharply stained spots were considered positive. Nonspecific esterase staining of MNC was performed by the method of Koski et al. (13), with α -naphthyl butyrate as substrate. Over 500 cells were counted, and samples of MNC with multiple intensely red-stained granules in the cytoplasm were considered positive.

Separation of adherent and nonadherent cells. MNC from peripheral blood or pleural fluid were suspended to 2×10^6 cells per ml in RPMI 1640 containing 10% heat-inactivated fetal bovine serum; 5 ml was transferred to plastic dishes (no. 3002; Falcon Plastics, Oxnard, Calif.) and incubated in humidified 5% CO₂ in air for 2 h at 37°C. After incubation, the dishes were shaken slowly, and nonadherent (NA) cells were removed. The plastic dishes were then washed five times with RPMI 1640, and adherent cells were obtained by dislodging the cells adhering to the dishes with a rubber policeman. Approximately 70% of adherent cells and less than 5% of NA cells stained positive by esterase staining.

Purification of T cells. T cells were purified by the combined method of SRBC rosette formation and nylon wool column filtration. Erythrocyte-rosetting MNC as described above were gently suspended, layered over Ficoll-Hypaque, and centrifuged for 20 min at $400 \times g$. The rosette-rich fraction was collected from the bottom of the tube, hemolyzed in 0.8% NH₄Cl, and then filtered through a nylon wool column by the method of Greaves and Brown (10). Cells passing through the column were designated as T cells. These T-cell fractions contained over 95% SRBC rosette-forming cells, less than 0.2% surface immunoglobulin-bearing cells, and less than 0.1% esterase stain-positive cells.

PPD and culture medium. PPD was kindly donated by H. Fujii, Institute for Microbial Disease, Osaka University. Culture medium consisted of RPMI 1640 supplemented with 10% pooled human AB serum, 100 μ g of streptomycin per ml, and 100 U of penicillin per ml. The same lot of PPD and human serum was used throughout the study.

PPD-induced proliferative response. Peripheral blood or pleural fluid MNC were cultured at 2×10^5 cells per 0.2 ml of culture medium in microculture plate (no. 3040; Falcon). The optimal culture period and final PPD concentration were 6 days and 50 μ g/ml, respectively. Cultures were performed in a humidified atmosphere at 37°C in 5% CO₂ in air and 0.2 μ Ci of

[³H]thymidine (specific activity, 5 Ci/mmol; Radiochemical Centre, Amersham, England) was added to each well 18 h before harvesting. All cultures were performed in triplicate. Cells were collected onto glass filter disks and washed with a semiautomated microharvesting device (Labo Mash; Labo Science Co., Ltd., Tokyo, Japan). ³H content was determined by a liquid scintillation spectrometer (Tri-Carb; Packard Instrument Co., Inc., Downers Grove, Ill.). Results were expressed as counts per minute (cpm) of [³H]thymidine incorporated per culture and as Δ cpm where Δ cpm = cpm of [³H]thymidine incorporated per stimulated culture - cpm of [³H]thymidine incorporated per unstimulated culture.

PPD-induced production of LMF. MNC were suspended at a concentration of 5×10^6 cells per ml in culture medium and were cultured in tissue culture tubes (no. 3033, Falcon) for 24 h at 37°C in a humidified atmosphere of 5% CO₂ in air. Cultures used for LMF production received 50 μ g of PPD per ml at the onset of the incubation period and were designated as P cultures. Control cultures received PPD (50 μ g/ml) at the end of the incubation period and were designated as R cultures. Incubation was stopped by pelleting the cells for 10 min at $2,000 \times g$. Culture supernatants were decanted from the cells, filtered through a 0.45- μ m Millipore filter (Millipore Corp., Bedford, Mass.) and stored at -20°C until tested. According to the preliminary investigations, the culture conditions described above were optimal for the production of LMF by MNC, and LMF activity in those culture supernatants remained constant for at least 6 months. Moreover, we confirmed that LMF production was antigen specific and LMF was produced by T cells.

Assay of LMF activity. Human peripheral blood B lymphocytes obtained from a single healthy donor were used as the indicator cells for the assay of LMF activity. B lymphocytes were obtained as a fraction of SRBC rosette-forming cell-depleted MNC. They contained less than 5% SRBC rosette-forming cells, and they were unable to proliferate by the stimulation with PPD (from 0.01 to 100 μ g/ml). These B lymphocytes were suspended at a concentration of 2×10^6 cells per ml in culture medium, and 0.1-ml aliquots were placed in each triplicate microculture plate well. Supernatants (0.1 ml) of P or R cultures were added to the cell suspensions. The optimal culture period was 6 days. [³H]thymidine (0.2 μ Ci) was added to each well 18 h before harvesting, and radioactivity incorporated to cells was counted by the method described in the proliferative response. LMF activity was calculated according to the following formula: LMF activity = cpm incorporated by B lymphocytes stimulated by P supernatants - cpm incorporated by B lymphocytes stimulated by R supernatants. The cpm incorporated by R supernatants were always less than 1,000.

Irradiation. Adherent cells suspended in culture medium were irradiated in vitro by exposure to X rays from a X-ray generator (STABILIPAN 300/2; Siemens-Reiniger-Werke AG, Erlangen, Germany).

Statistical analysis. The significance of the difference between groups was calculated by Student's *t* test.

RESULTS

Cell characterization of MNC in peripheral blood and pleural fluid. MNC in pleural fluid and

TABLE 1. Characterization of MNC from the peripheral blood and pleural fluid in patients with tuberculous pleurisy

Source of MNC	No. of subjects	% SRBC rosette-forming cells	% Surface immunoglobulin-bearing cells	% Nonspecific esterase stain-positive cells
Peripheral blood	9	68.9 ± 2.2 ^a	14.0 ± 2.6	13.7 ± 1.8
Pleural fluid	9	88.4 ± 1.7 ^b	4.8 ± 1.0 ^b	1.4 ± 0.7 ^b

^a Mean ± standard error of the mean.

^b Significantly different from peripheral blood; $P < 0.001$.

MNC in peripheral blood of patients were obtained on the same day. Based on morphological studies, pleural fluid contained greater than 90% lymphocytes. These MNC were employed for rosette formation with SRBC, detection of surface immunoglobulin determinants, and nonspecific esterase staining. The data in Table 1 show that the percentage of SRBC rosette-forming cells (T cells) was significantly higher in pleural fluid MNC than in peripheral blood MNC, whereas surface immunoglobulin-bearing cells (B cells) and esterase stain-positive cells (macrophages) in pleural fluid MNC were less than in peripheral blood MNC ($P < 0.001$). The percentages of T cells, B cells, and macrophages in peripheral blood MNC from these patients were similar to those in peripheral blood MNC from healthy controls.

PPD-induced proliferative response and LMF production. The PPD dose-response curves and kinetic studies revealed the highest proliferation in cultivation of both pleural fluid and peripheral blood MNC for 6 days with a PPD dose of 50 µg/ml. Peripheral blood MNC from patients with tuberculous pleurisy responded significantly less than those from pulmonary tuberculosis patients without effusion and healthy controls in the proliferative response. These decreased responses in pleurisy patients were not due to increased "baselines," because the mean values of [³H]thymidine incorporation per unstimulated culture were 944 cpm in the pleurisy group and

833 cpm in the control group. On the contrary, pleural fluid MNC from patients with tuberculous pleurisy were significantly more responsive than peripheral blood MNC from the pleurisy patients, pulmonary tuberculosis patients, and healthy controls (Table 2). Responses of peripheral blood and pleural fluid MNC obtained from each patient on the same day and assayed simultaneously are shown in Fig. 1. Pleural fluid MNC from 17 of 22 patients had greater responsiveness than peripheral blood MNC in the proliferative response (Fig. 1). The results of the LMF production paralleled those of the proliferative response. Peripheral blood MNC from patients with tuberculous pleurisy showed less LMF production than those from other groups, and pleural fluid MNC produced LMF more than peripheral blood MNC from other groups (Table 3, Fig. 2).

Response of NA cells. The effect of depletion of adherent cells from MNC was examined in the PPD-induced proliferative response (Fig. 3). Cells were cultured with 50 µg of PPD per ml for 6 days. NA cells responded more than unfractionated MNC (UF cells) in the peripheral blood MNC from 13/16 (81%) patients with tuberculous pleurisy. Moreover, the mean value of [³H]thymidine incorporation by these NA cells of $(35.1 \pm 3.1) \times 10^3$ cpm was significantly greater than that by UF cells of $(19.9 \pm 2.3) \times 10^3$ cpm ($P < 0.01$). NA cells responded over UF cells in the peripheral blood MNC from 5 of

TABLE 2. PPD-induced proliferative response of MNC from tuberculous patients^a

Subjects	Source of MNC	No. of subjects	[³ H]thymidine incorporated (10 ³ Δcpm) mean ± SE
Tuberculous pleurisy	Peripheral blood	27	19.1 ± 2.5 ^b
	Pleural fluid	23	36.4 ± 2.8 ^c
Pulmonary tuberculosis	Peripheral blood	30	30.5 ± 1.9
Healthy control ^d	Peripheral blood	41	30.7 ± 1.7

^a MNC from peripheral blood or pleural fluid were cultured with 50 µg of PPD per ml for 6 days. [³H]thymidine incorporated during the last 18 h was counted.

^b Significantly different from healthy control and pulmonary tuberculosis, $P < 0.05$.

^c Significantly different from peripheral blood of tuberculous pleurisy, $P < 0.01$. Significantly different from healthy control and pulmonary tuberculosis, $P < 0.05$.

^d Positive for tuberculin skin test.

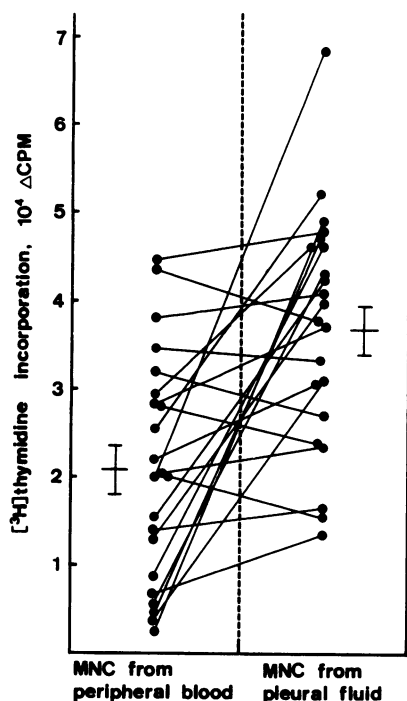


FIG. 1. PPD-induced proliferative response of MNC from peripheral blood and pleural fluid. Each point connected by a line indicates the response from the same individual. Peripheral blood was collected on the day of collecting pleural fluid. MNC were cultured with 50 μg of PPD per ml for 6 days. [^3H]thymidine incorporated during the last 18 h was counted. The vertical bars represent the mean \pm standard error of the mean.

12 (42%) patients with pulmonary tuberculosis and 7 of 17 (41%) healthy controls; the mean response of NA cells was not significantly different from that of UF cells in those subjects, nor was the response of NA cells significantly different from that of UF cells in pleural fluid MNC. Figure 4 shows the kinetics of the proliferative

response of UF and NA cells stimulated with 50 μg of PPD per ml and Fig. 5 shows dose-response curves of those cells stimulated with PPD in the 6-day culture. The increased response of proliferation after depletion of adherent cells was observed only in peripheral blood MNC from patients with tuberculous pleurisy. Table 4 shows PPD-induced LMF production as well as PPD-induced proliferative response of both UF and NA cells from peripheral blood MNC from three patients with tuberculous pleurisy. NA cells showed greater LMF production than UF cells; these results were in parallel with those of the proliferative response.

Response of T cells. To compare the reactivity of T cells in pleural fluid with that of T cells in peripheral blood, 2×10^5 T cells purified from pleural fluid MNC from pleurisy patients or those from peripheral blood MNC from the same patient were reconstituted with various numbers of autologous adherent cells which had been obtained from peripheral blood MNC and irradiated with 2,000 rad. These T cells were cultured with 50 μg of PPD per ml for 6 days, and [^3H]thymidine incorporated during the last 18 h was counted. Representative results are shown in Fig. 6. Pleural fluid T cells showed greater response than peripheral blood T cells from the same patient irrespective of the numbers of adherent cells added (Fig. 6). The data in Fig. 6 further indicate that the addition of increasing numbers of adherent cells suppressed the PPD-induced proliferation of T cells from both pleural fluid and peripheral blood.

DISCUSSION

Our study showed that pleural fluid MNC were predominantly T cells and highly responsive to in vitro stimulation with PPD. In contrast, peripheral blood MNC of these patients were less responsive even than those of tuberculin-positive healthy individuals. We showed that responsiveness of patients' peripheral blood

TABLE 3. LMF production by MNC from tuberculous patients^a

Subjects	Source of MNC	No. of subjects	LMF activity ^b (10^3 cpm) mean \pm SE
Tuberculous pleurisy	Peripheral blood	17	3.31 \pm 0.53 ^c
	Pleural fluid	12	19.3 \pm 2.1 ^d
Pulmonary tuberculosis	Peripheral blood	20	5.07 \pm 0.41
Healthy control ^e	Peripheral blood	28	7.36 \pm 0.70

^a MNC from peripheral blood or pleural fluid were cultured with 50 μg of PPD per ml for 24 h.

^b Human peripheral blood B lymphocytes obtained from a single healthy donor were used as the indicator cells for the assay of LMF activity.

^c Significantly different from pulmonary tuberculosis, $P < 0.02$. Significantly different from healthy control, $P < 0.001$.

^d Significantly different from other three groups, $P < 0.001$.

^e Positive for tuberculin skin test.

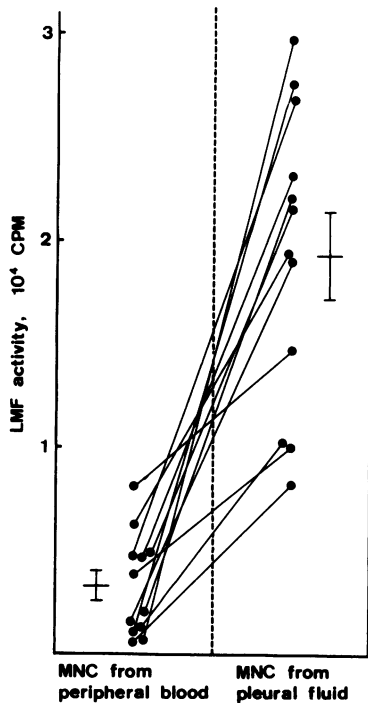


FIG. 2. LMF production by MNC from peripheral blood and pleural fluid. Each point connected by a line indicates the response from the same individual. Peripheral blood was collected on the day of collecting pleural fluid. MNC were cultured with 50 μ g of PPD per ml for 24 h to produce LMF. Human peripheral blood B lymphocytes obtained from a single healthy donor were used as the indicator cells for the assay of LMF activity. The vertical bars represent the mean \pm standard error of the mean.

MNC to PPD stimulation was restored by the elimination of adherent cells in MNC. These results suggest that the hyporesponsiveness of peripheral blood MNC from pleurisy patients was not due to a decline in the function of T-cell themselves, but to the effect of suppressor cells. These suppressor cells may be activated macrophages, since they have the ability to adhere rigidly, and pleural fluid MNC, which contain few macrophages, were highly responsive. It is known that activated macrophages have the capacity to inhibit the T-cell function (19, 20, 23, 24). On the other hand, as macrophages were required to activate T cells by soluble antigen such as PPD (2) and, as shown in our study, the number of macrophages affected the magnitude of T-cell response (Fig. 6), it is conceivable that the hyporesponsiveness of peripheral blood MNC from pleurisy patients and the recovery of the response by the elimination of adherent cells were simply due to the percentage of macro-

phages involved. This is, however, unlikely because the percentage of esterase-positive cells in peripheral blood MNC from pleurisy patients was similar to that in peripheral blood MNC from healthy controls (Table 1). Ellner (5) showed the existence of suppressor macrophages in the peripheral blood from tuberculous patients with low tuberculin responses. Our data suggested that suppressor macrophages were present in the peripheral blood from pleurisy patients who responded to PPD within normal range as well as in low responders, although the differences in nonadherent cell responses might reflect different efficiencies of removal of adherent cells on plastic dishes or different degrees of accessory function of residual macrophages or both (Fig. 3). Suppressor macrophages may be responsible for the depressed manifestation of delayed hypersensitivity observed in patients with pleurisy.

In regard to the antigen specificity of the depressed response of peripheral blood MNC in the pleurisy patients, we did not examine other antigens in this study. However, peripheral

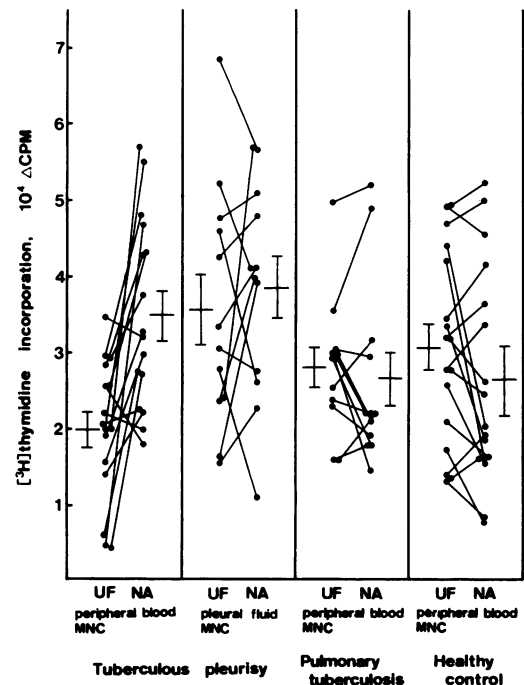


FIG. 3. PPD-induced proliferative response of UF MNC and NA cells from peripheral blood or pleural fluid. Each point connected by a line indicates the response from the same individual. UF and NA cells were cultured with 50 μ g of PPD per ml for 6 days. [3 H]thymidine incorporated during the last 18 h was counted. The vertical bars represent the mean \pm standard error of the mean.

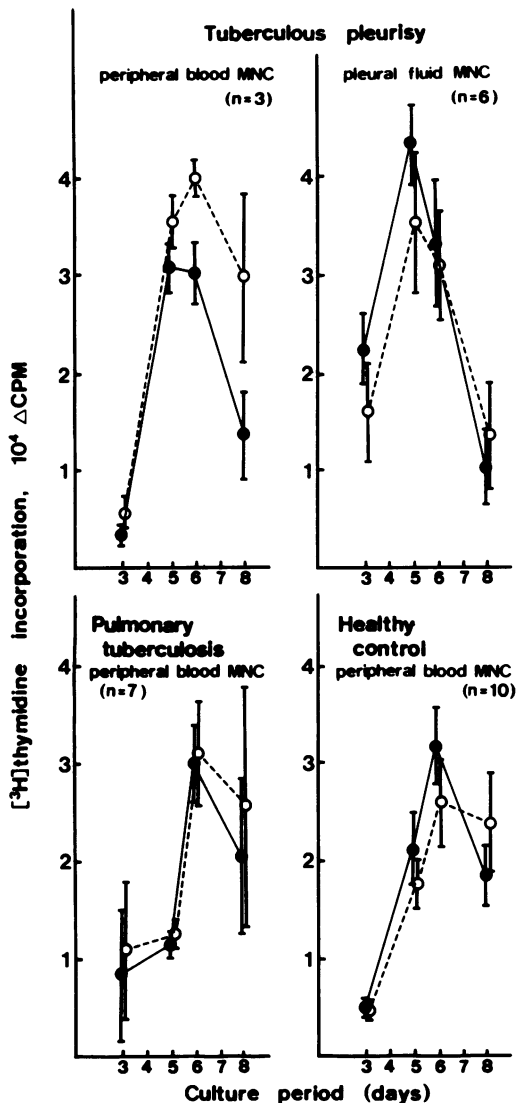


FIG. 4. Kinetics of the proliferative response of UF MNC and NA cells stimulated with PPD. UF (●) and NA (○) cells from peripheral blood or pleural fluid were cultured with 50 μg of PPD per ml for various periods. ³H]thymidine incorporated during the last 18 h was counted. Vertical bars represent the standard error of the mean.

blood MNC from some pleurisy patients which responded poorly to PPD stimulation gave proliferation to phytohemagglutinin, a polyclonal T cell activator, as high as those from healthy controls (data not shown). Results of PPD-induced LMF production of peripheral blood and pleural fluid MNC from pleurisy patients were in parallel with those of the proliferative response (Table 3, Fig. 2); NA cells from peripheral blood

in the pleurisy patients showed greater LMF production than unfractionated blood MNC (Table 4). These observations suggest that suppressor macrophages are responsible for the suppression of the lymphokine production of T cells as well as DNA synthesis.

The data in Fig. 6 indicate that pleural fluid T cells responded to PPD stimulation more than peripheral blood T cells from the same patient at any point of addition of adherent cells, which is suggesting that more PPD-reactive T cells were

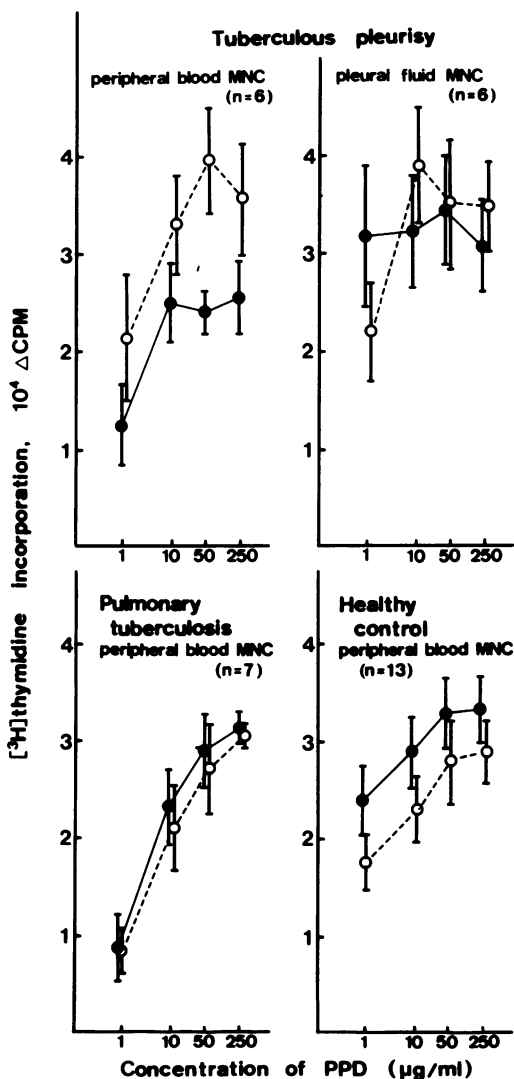


FIG. 5. Dose-response curves of UF MNC and NA cells stimulated with PPD. UF (●) and NA (○) cells from peripheral blood or pleural fluid were cultured for 6 days in the presence of various concentrations of PPD. ³H]thymidine incorporated during the last 18 h was counted. Vertical bars represent the standard error of the mean.

TABLE 4. Effect of adherent cell-depletion on the PPD-induced LMF production^a and proliferative response of peripheral blood MNC from patients with tuberculous pleurisy

Patient	LMF activity ^b (cpm)		Proliferative response ^c (Δcpm)	
	UF	NA	UF	NA
1	4,921	19,729	19,999	55,141
2	6,007	8,686	4,668	46,784
3	2,006	3,082	13,885	22,158

^a MNC were cultured with 50 μg of PPD per ml for 24 h to produce LMF.

^b Human peripheral blood B lymphocytes obtained from a single healthy donor were used as the indicator cells for the assay of LMF activity.

^c MNC were cultured with 50 μg of PPD per ml for 6 days. [³H]thymidine incorporated during the last 18 h was counted.

accumulated in pleural fluid than in peripheral blood. Moreover, the addition of increasing numbers of adherent cells suppressed the response of T cells from both pleural fluid and

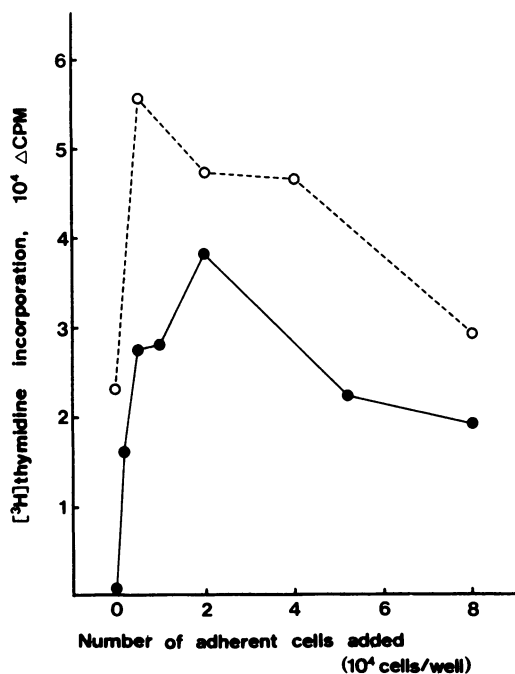


FIG. 6. PPD-induced proliferative response of T cells from a patient with tuberculous pleurisy. T cells from pleural fluid (O) and peripheral blood (●) mononuclear cells from the same patient were obtained and reconstituted with various numbers of autologous adherent cells. T cells (2×10^5 /well) were cultured with 50 μg of PPD per ml for 6 days. [³H]thymidine incorporated during the last 18 h was counted. Adherent cells were obtained from peripheral blood MNC and irradiated ($2,000$ rad).

peripheral blood. Taken together those observations, hyperresponsiveness of pleural fluid MNC to PPD stimulation may be caused by more PPD-sensitive T cells or fewer adherent cells containing the suppressor macrophages (or both) in pleural fluid as compared with peripheral blood. It is conceivable that large numbers of highly PPD-reactive T cells were recruited to the pleural fluid from peripheral blood to proliferate by the stimulation of tubercle bacilli or related antigens.

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