CHAPTER II

MATERIALS AND METHODS

Sample and specimen collection.

A. Patients

Thirty patients with pleural effusion on chest X-ray from the Chest Service of the Department of Medicine, Chulalongkorn Hospital; Central Chest Hospital, Nondhaburi Province; National Cancer Institute of Thailand and Bangkok Central Chest Clinic, Division of Communicable Disease Control, Ministry of Public Health were studied. The patients were categorized into 3 groups : tuberculous, non-tuberculous and non-specific groups according to the following criteria.

1. Tuberculous group

The patients in this group were diagnosed to have tuberculous effusion according to the following criteria:

1.1 Major criteria:

Pleural fluid positive acid fast bacilli (AFB) found, or positive culture for Mycobacterium tuberculosis.

1.2 Minor criteria:

1.2.1 Exudative pleural fluid effusion (protein over 3 gm% and specific gravity over 1.016) with mononuclear cell pleocytosis.

1.2.2 Concomitant lung parenchyma lesion on X-ray.

1.2.3 Positive tuberculin test.

1.2.4 Pleural biopsy showed granulomatous lesion with or without caseous necrosis.

1.2.5 Clinical response to anti-tuberculous treatment within 3 mounts.

A diagnosis of tuberculous effusion was made if the patients had one major or 4 out of the 5 minor criteria.

2. Non-tuberculous group.

This group consisted of patients with mononuclear cell pleural effusions who did not meet the above criteria of tuberculous effusion but who had other proven causes of pleural effusion such as malignancy, congestive heart failure, chirhosis, systemic lupus erythrematosus, etc.

3. Non-specific group.

This group consisted of patients who did not fulfil the criteria of tuberculous effusion or had other obvious causes for a pleural effusion in group 2.

B. Specimen collection.

On the day of pleural tapping, the pleural fluid was collected for routine laboratory investigations i.e. AFB smear, gram stain, culture and sensitivity for tuberculosis, cytologic examination, cell count, protein content, etc. Another 50 ml. of pleural fluid were collected in sterile heparinized flasks containing 0.3 ml. of 5000 I.U./ml. heparin (Leo, Bellerup, Denmark) to give a final concentration of 30 I.U.heparin /millilitre of pleural fluid with thorough mixing. Simultaneously, 20 ml. of heparinized venous blood containing heparin 25 I.U./ml. was collected. The heparinized pleural fluid and blood were obtained for immunologic studies as outlined in Figure 1 and 2.

As shown in Figure 1, heparinized pleural fluid was examined for total leukocyte count, differential cell count and counting for non-specific esterase staining cell representing macrophage and monocyte. After centrifugation, cell-free supernatant pleural fluids were tested for free lymphokines namely, leukocyte migration inhibition factor (LIF) by indirect leukocyte migration inhibition factor test (LIF test). The cell pellets were resuspended in RPMI 1640 (Gibco, Grand Island, N.Y., USA.) and pleural fluid mononuclear cells (PFMC) were isolated by Ficoll-Hypaque density gradient centrifugation (Figure 3). These PFMC were

enumerated for total T-cells, B-cells and T-cell subsets according to the methods described below. Furthermore, these mononuclear cells were tested for proliferative response to purified protein derivative (PPD) and for direct LIF test in response to PPD.

As shown in Figure 2, heparinized blood was also analysed for total white blood cell count and differential cell count. Peripheral blood mononuclear cells(PBMC) were isolated from 10 ml. of heparinized blood by Ficoll-Hypaque density gradient centrifugation. The PBMC were enumerated for T-cells, B-cells, T-cell subsets and analysed for proliferative response to PPD as was done with the PFMC.

C. Analysis of final results.

These patients had been followed for up to 3 months by the clinicians. Clinical data which concluded the diagnosis of TB or non-TB, pleural effusion were compared with the results from the immunologic studies in a double-blind fasion.

D. Healthy donor control group.

Heparinized blood from healthy blood donors at the National Blood Bank Centre of the Thai Red Cross Society was used as normal controls in the study.

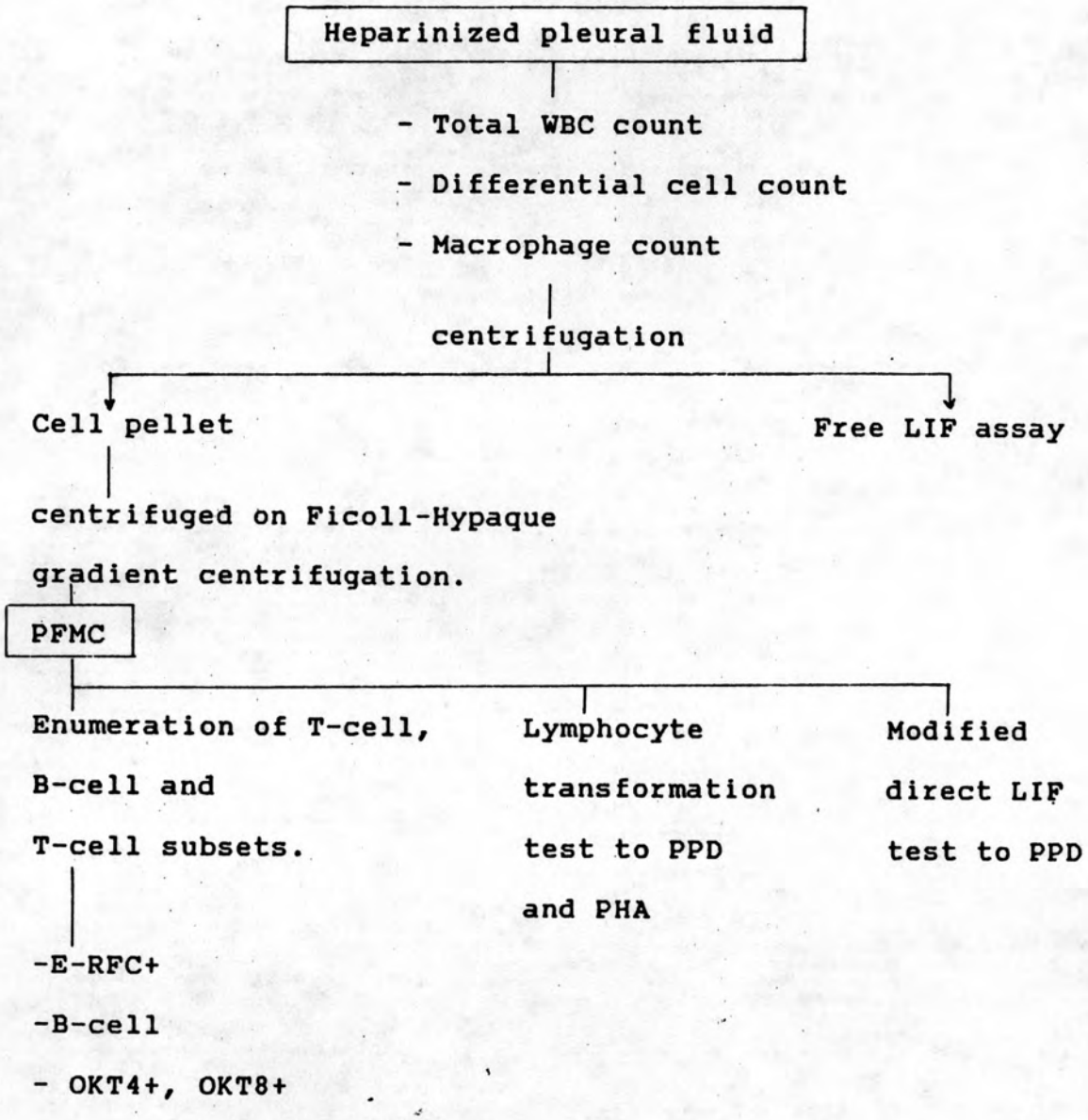

Figure 1. Protocol of immunologic study of pleural fluid.

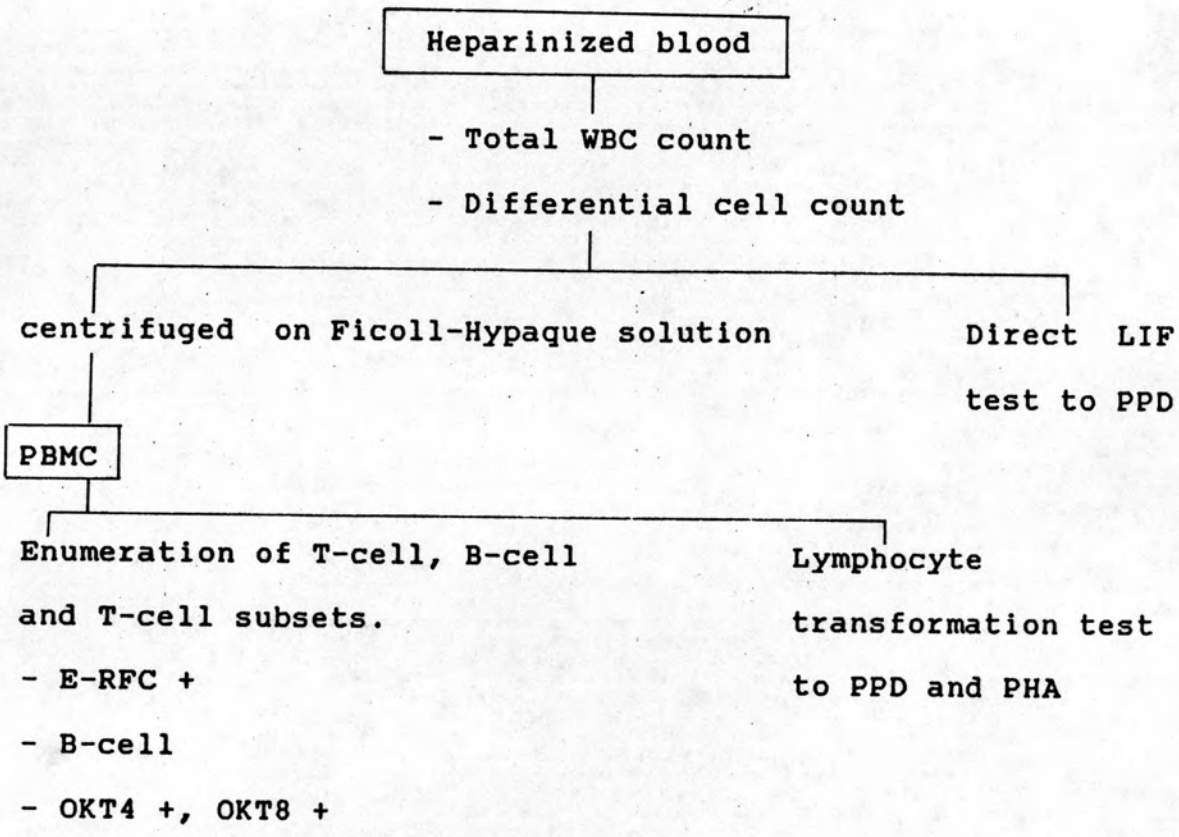

Figure 2. Protocol of immunologic study of heparinized blood.

Characterization of lymphocytes and lymphocyte subpopulations

1. White blood cell count and differential cell count.

Heparinized pleural fluid and heparinized blood were diluted 1:20 with white blood cell diluent (see, Appendix II) in a white blood cell diluting pipette, mixed and dropped into a Newbour hemocytometic chamber (American Optic, USA.). The white blood cells were counted in the 4 large square white blood cell areas and calculated as the number of leukocytes/cu.mm. (47). A drop of heparinized blood or pleural fluid cell pellet placed on glass slide was spread with the edge of another glass slide and allowed to dry at room air. The blood films were then stained with Wright's stain (see, Appendix II). The different types of leukocytes in 100 white blood cells were enumerated under the microscope.

2. Collection of pleural fluid leukocytes.

Heparinized pleural fluid was aliquoted in sterile tubes and centrifuged at 1800 rpm. (200 G) for 10 minutes at 4 C. Pleural fluid supernatant was collected and stored at -70 C for free lymphokine assay. The cell pellet was resuspended in 10 ml. of RPMI 1640 before subjected to lymphocyte separation.

3. Separation of lyphocytes from blood and pleural fluid.

Pleural fluid mononuclear cells (PFMC) or peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation on Ficoll-Hypaque solution (see, Appendix II)(48). Four ml. of heparinized blood or 5 ml. of pleural fluid leukocyte suspension were overlayered on 3 ml. of Ficoll-Hypaque solution by aseptic technique and centrifuged at 220 G for 30 mins. at 20 C. The mononuclear cells (MNC) at Ficoll-Hypaque-Plasma interface were collected by the use of sterile pasture pipettes (Figure 3). The cells were washed twice with 5 ml. of Hank's Balanced Salt solution (HBSS)(Gibco, Grand Island, N.Y., USA.) by centrifugation at 1800 rpm. (200 G), 4 C for 5 mins. After the secound wash, the MNC pellet was resuspended in 5 ml. RPMI 1640 and recentrifuged. The cell pellet was resuspended in tissue culture medium (TCM) which was RPMI 1640 subplemented with 5% heat inactivated pooled human AB serum, 100 Ug/ml. streptomycin (Dumex, Bangkok, Thailand), 100 Units/ml. penicillin (Dumex, Bangkok, Thailand) and 10 mM HEPES (Sigma, Mo., USA.) (see, Appendix II). The cells were counted in a hemocytometer and adjusted to 2×10^6 cells/ml. with TCM. These PFMC or PBMC suspensions contained approximately 95-98% lymphocytes, 1-4% monocytes and 0-2% polymorphonuclear cells as examined on Wright's stain and 95-98% of these

cells were viable as determined by trypan blue exclusion (see, Appendix II).

4. Spontaneous E-rosette formation.

T-cell numbers was determined by spontaneous E-rosette formation technique described by Hoffman and Kunkel (49) with slight modification. The PBMC or PFMC suspensions at $2x10^6$ cells/ml. were mixed in equal volumes (0.5 ml. each) with 1% sheep red blood cell suspension (SRBC)(see, Appendix II) in RPMI 1640 in a 12x75 mm. sterile test tube and incubated at 37 C for 5 mins. After the initial incubation, the tubes were centrifuged at 1000 rpm. (150 G) for 5 mins. at 4 C and let stand overnight in a refrigerator at 4 C. After overnight incubation, the cell pellets were very gently resuspended by tipping the tube and slowly twisting it, and a few drops of the cell suspensions were transfered to a hemocytometer chamber. After the cells were allowed to sediment, E-rosette forming cells (E-RFC+) as defined by the cells surrounded by three or more SRBC (Figure 4), were scored under the light microscope from a total of 200 mononuclear cell and the percentage of E-RFC+ cells was calculated.

5. Enumeration of T-cell subpopulations.

The monoclonal antibodies directed to human T-cell surface antigens were used to classify human T-lymphocyte subpopulations (50,51). OKT4+ and OKT8+ cells representing human inducer/helper and suppressor/cytotoxic T-cells respectively were determined by indirect immunofluorescent technique using OKT4 and OKT8 monoclonal antibodies (Ortho Diagnostic, N.J., USA.). $1x10^6$ PBMC or PFMC suspensions were spinned at 1800 rpm. (200 G) for 5 mins. to remove the supernatant. 5 Ul. of undiluted monoclonal antibodies was added to the cell pellet, mixed and incubated at 4°C or in the ice bath for 30 mins. with gentle agitation every 10 mins. After incubation, 2 ml. of HBSS was added to the cell suspension and the cells were washed 2 more times by centrifugation at 1800 rpm. (200 G), 4°C for 5 mins. After the last wash, the cell pellet was mixed with 100 Ul. of fluorescien-conjugated rabbit anti-mouse immunoglobulin (Dako immunoglobulins, Copenhagen, Denmark) at 1:20 dilution and incubated 30 mins. at 4°C or in the ice bath with thorough shaking every 10 mins. This was followed by 3 washes in phosphate buffer saline (PBS), pH 7.4 (see, Appendix II). The cell pellet was preserved in 0.5 ml. of paraformaldehyde (Sigma, Mo., USA.) in PBS (see, Appendix II). The cells were mixed and again incubated in the ice bath or at 4°C

for 10 mins. After centrifugation at 1800 rpm. for 10 mins., the preserved cell pellet was resuspended in one drop of mounting media (see, Appendix II) followed by vigorous mixing. The cells were then mounted on a microscopic glass slide with cover slip for fluorescence microscopic examination. The fluorescence-stained lymphocytes (Figure 5) were counted in a total of at least 300 lymphocytes visualized on bright light field and scored as the percentage of OKT4+ or OKT8+ cells respectivly.

6. Enumeration of B-cells

B-lymphocyte were enumerated by direct immunofluorescence staining of their surface membrane immunoglobulins according to the method of Winchester and Fu (52). $1x10^6$ cells in the PBMC or PFMC suspension were centrifuged at 1800 rpm. (200 G), 4 C for 5 mins. to remove the supernatant. 0.1 ml. of fluorescein-labelled sheep antihuman Ig G, A, M polyvalent specific (Wellcome, Beckenhem, UK.) at 1:20 dilution was added to the cell pellet, followed by gentle mixing and incubation at 4 C or in ice bath for 30 mins. with frequent shaking at 10 mins. interval. After two washings in PBS pH 7.4 at 1800 rpm. (200 G), 4 C for 5 mins., the cells were preserved with 0.5 ml. of 1% paraformaldehyde in PBS and incubated at 4 C for 5 mins. The cells were then spinned at 1800 rpm.

(200 G), 4 C for 5 mins. and mounted with a drop of mounting media for fluorescence microscopy. The percentage of membrane immunoglobulin staining cells was scored from a total of 300 lymphocytes.

7. Identification of monocytes and macrophages in pleural fluid.

One useful histochemical reaction for identification of macrophages and monocytes from within other cells is the staining technique for nonspecific esterase enzyme (53). The staining technique for nonspecific esterase originally described by Yam et al (54) and modified by Koski et al (55) was used. The cell pellet from pleural fluid after centrifugation was smeared on a glass slide and air dried. The slide was fixed in cold fixative solution (see,Appendix II) for 30 seconds and rinsed by transferring the slide through four jars of distilled water and air dried. 1 ml. of fluted filter paper-filtrated pararosanilline (Sigma, Mo., USA.) solution (see, Appendix II) was mixed with 1 ml. of freshly prepared 4% sodium nitrite (BDH, Poole, UK.) (see, Appendix II) and let stand for 1 min. before used in order to allow for complete hexazotization. 44.5 ml. of M/15 phosphate buffer, pH 6.3 (see, Appendix II); hexazotization paralosaniline, 0.25 ml.; respectivly and filtered into couplin jar. The dried slide was dipped in

the couplin jar and incubated at 37 C in a water bath for 45 mins. The slide was then rinsed and counter-stained with 0.5% methyl green (BDH, Poole, UK.) (see, Appendix II) for 15 mins. The air-dried slide was mounted with Permount (Fisher scientific, USA.) for microscopic examination. Nonspecific esterase enzyme positive cells (NSE+) showed multiple intensely red-stained granules in the cytoplasm compared with the green counter stain of the esterase-negative cells (NSE-)(Figure 6). The percentage of NSE+ cells were counted in a total of 100 leukocytes from the pleural fluid.

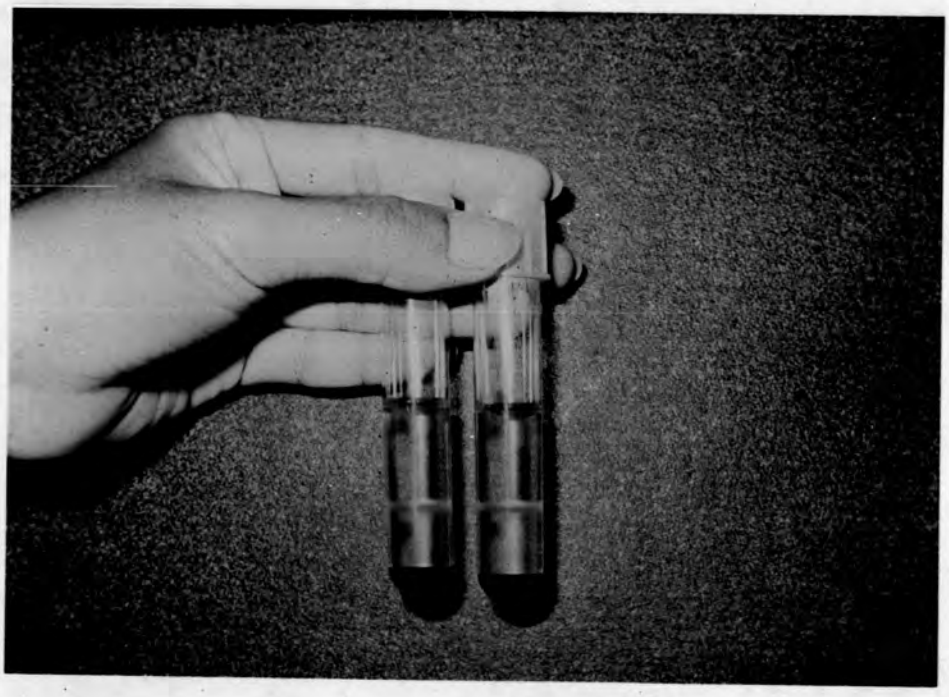

Figure 3. Mononuclear cell layer on Ficoll-Hypaque gradient centrifugation.

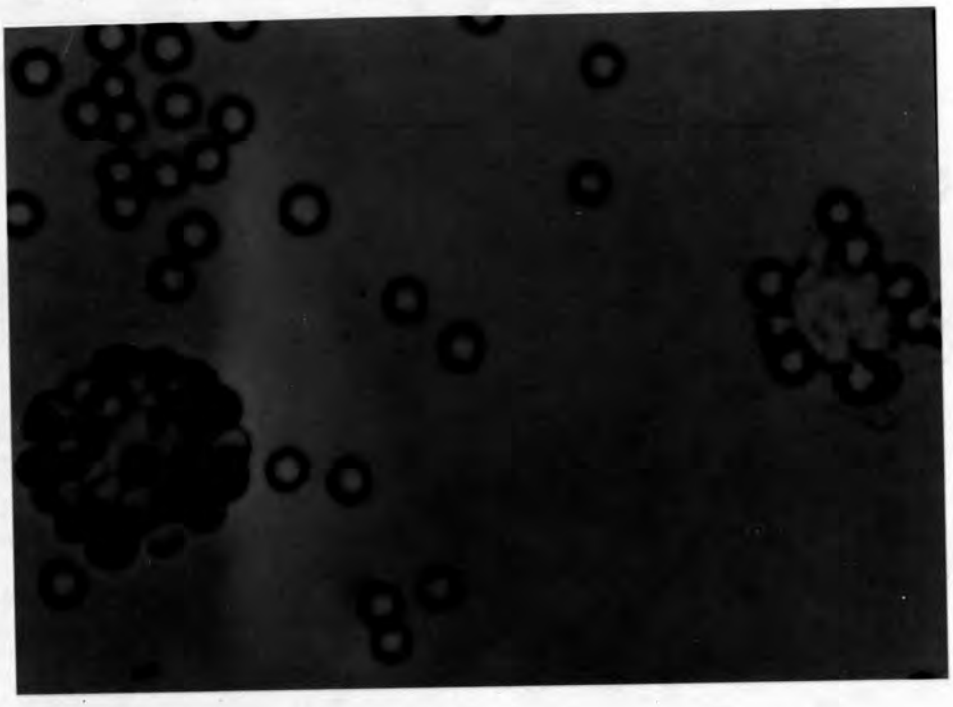

Figure 4. E-RFC+ cells.

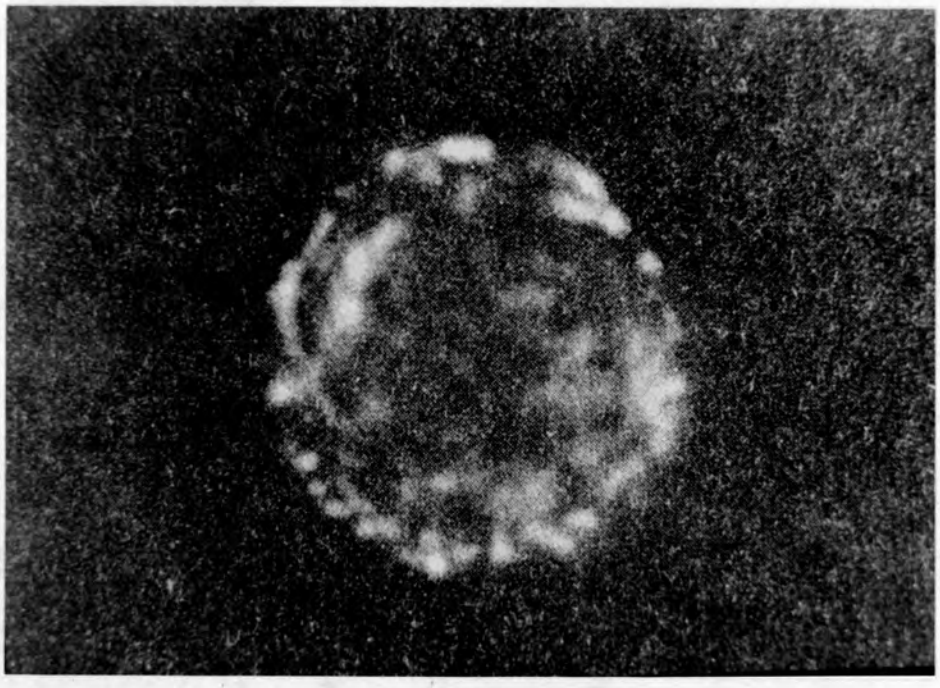

Figure 5. Immunofluorescent staing positive cells.

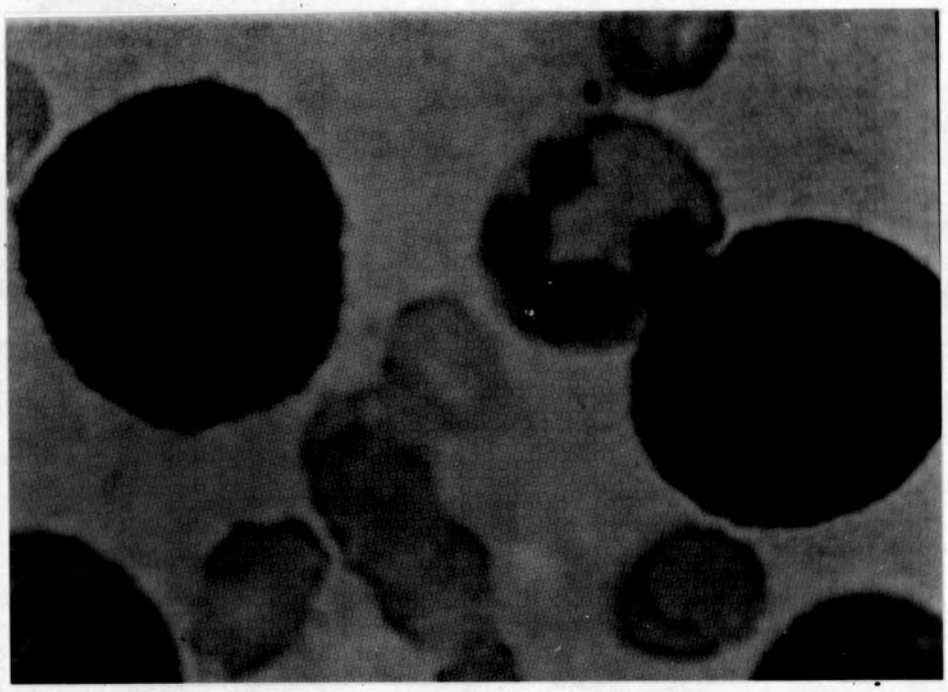

Figure 6. Non-specific esterase staing positive cells. (red color)

Leukocyte Migration Inhibition Factor Test (LIF test).

1. Tuberculin induced migration inhibition of peripheral blood leukocytes in agarose medium.

The direct leukocyte migration inhibition factor test in agarose medium was performed according to the methods of Clausen (57) and Bendixson (58) with partial modification. 10 ml. of heparinized blood was mixed with 2 ml. of 6% dextran (Pharmacia Fine, Upsala, Sweden) in normal saline solution (see, AppendixII) and let stand at 37 C for 1 hour. After red blood cells sedimented to the bottom of the tube, the leukocyte rich plasma was removed to a 80X60 mm. polystyrene conical base test tube (Nunc, Roskilde, Denmark.) with sterile Pasture pipette and centrifuged at 1200 rpm. for 5 minutes. The supernatant plasma was tipped off and the cell pellet was resuspended in HBSS and washed three times by centrifugation at 1200 rpm. for 5 min. in HBSS. After the third wash, leukocyte pellets were resuspended in TC 199 medium containing TC 199 (Difco, Michigan, USA.) supplemented with 10% horse serum (Flow Lab., Ayrshire, Scottland.), 10 mM. HEPES , 100 Units / ml. penicillin G and 100 Ug / ml. streptomycin pH 7.4 (see, Appendix II). The cell suspension was counted and adjusted to give a leukocyte concentration of 2.4×10^{8} cells / ml. by diluting with TC 199 medium. 90 Ul. of this leukocyte

suspension were mixed with 10 Ul. 0f 1000 Ug / ml. of purified protein derivative (PPD)(Staten seruminstitut, Copenhagen, Denmark). Such an incubation mixture gave a final concentration of leukocytes 2.2 X 10^8 cells / ml. which was stimulated with PPD 100 Ug / ml. As a control condition, 90 Ul. of leukocyte suspension were mixed with 10 Ul. of TC 199 medium without PPD. After 1 hour incubation in a 37 C 5% CO_2 incubator with thorough mixing every 15 mins., the leukocyte mixtures were allowed to migrate in 0.8% agarose-TC 199 medium (see, Appendix II) placed in a 60 mm. diameter plastic petri dishes (Nunc, Intermed, Roslde, Denmark). 10 Ul. of the cell suspensions 10 Ul. were placed in the 3 mm. well punched in the agarose gel. The PPD stimulated and unstimulated leukocyte cultures were done in triplicate. The agarose plates were incubated at 37 C in a humidified atmosphere of 5% CO_2 for 18 hrs.. At the end of incubation, the plates were fixed with fixing reagent (see, Appendix II) for 3 hrs., washed with running tap water, and the agarose was slipped off from the plate. The leukocytes at the bottom of the well migrated out in the capillary cleft beneath the gel were fixed on the plastic plate and let air dry (figure 7). To determine the migrating inhibitory activity of specific antigen stimulation, the result was expressed as Migration Index (M.I.) which was the ratio of the migration area of PPD stimulated culture to that of the

same control culture.

$$\text{Migration Index (M.I.)} = \frac{\text{migration area of PPD stim. culture}}{\text{migration area of unstim. culture}}$$

The migration area was calculated from the diameter of migration area around the wells, measured by the calibrating veiwer TG model 2743 (Transidyne General, Michigan, USA.). The diameter of the migration zone of each well was averaged from two diameters measured from the longest and the shortest migratory borders. The average of the triplicate cultures was calculated and substracted by the circular area of the 3 mm. hole of the gel puncher (No. 51466, Gelman, USA.). The net migration areas from PPD stimulated and PPD-unstimulated cultures were then used to calculate M.I..

2. Direct LIF test of pleural fluid cells-the pilot study

The two pilot experiments of direct LIF test using whole pleural fluid cells from tuberculous patients according to the method described above for peripheral blood leukocytes showed that the predominant mononuclear cells contained in the pleural fluid could not migrate out of the holes of the agarose medium. There were some polymorphonuclear cells migrating beneath the gel, but it was impossible to measure the migratory zone. Therefore, in order to detect the LIF production from antigen-stimulated

pleural fluid lymphocytes, the test system had to be modified by adding purified human peripheral blood polymorphonuclear cells as target cells for LIF activity.

3. Purification of human peripheral blood granulocyte

Purified human peripheral blood polymorphonuclear cells (PMN) were prepared according to the method of Clausen (90). Four ml. of leukocyte-riched plasma from heparinized blood of healthy donors after sedimentation with 6% dextran in normal saline solution were overlayered on 3 ml. of Ficoll-Hypaque solution (see, Appendix II). After centrifugation at 400 G for 30 min. , the mononuclear cells in the interphase of the plasma and the Ficoll-Hypaque solution were carefully removed. The PMN cells at the bottom of the tube were collected and transfered to another tube by Pasture pipette. The adhearing PMN cells at the bottom of the tube were rubbed by sterile rubber policeman and pooled. After the second wash by centrifugation at 1200 rpm. 4 C for 5 min. with HBSS, the contaminated red blood cells were lysed by lysing solution (see, Appendix II). After the last washing, the PMN pellets were resuspended in TC 199 medium and counted (see, Appendix II). The PMN cells were smeared on the glass slide and stained with Wright 's stain (see, Appendix II). The PMN preperation contained 95-99% of PMN cells with 1-5% of contaminating mononuclear cells.

4. Modified direct LIF test of pleural fluid cells in agarose medium.

The isolated PFMC were washed twice in RPMI 1640 by centrifugation at 1800 rpm. for 5 minutes and resuspended in TC 199 medium (see, Appendix II) and adjusted to the final concentration of 1.5×10^6 cells/ml. On the same day of experiment, the allogeneic purified PMN suspension from healthy donors were also obtained by the method described above and adjusted by TC 199 medium to 4.4×10^8 cells/ml. The patients' PFMC were mixed with allogeneic purified PMN at a ratio of 1:3 (PFMC:PMN). The cell mixtures were incubated with PPD at a final concentration of 100 Ug/ml. or with TC199 medium alone as negative control at 37 C 5% CO_2 for 1 hour with mixing every 15 minutes. In addition, 2.2×10^8 PMN/ml. were incubated with and without 100 Ug/ml. PPD as a migratory cell control. At the end of incubation, the cells mixtures were dropped into the wells in the TC 199 agarose medium. After incubating the agarose culture plate at 37 C 5% CO_2 in the moist chamber for 18 hours, the plate was fixed with fixing agent (see, Appendix II) as described above and the Migration Index was determined. The value of the Migration Index of the experiment was accepted if the M.I. of PMN control was more than 0.8 .

5. Free LIF assay of pleural fluid supernatant.

Pleural fluid supernatents were assayed for free LIF which might be released in vivo by the indirect LIF test described by Clausen (57) with slight modification. Cell-free pleural fluid supernatants were inactivated at 56°C for 30 min. Pooled leukocyte-riched plasma from group O healthy donors was obtained by the method described above. The pooled leukocyte suspensions were washed 2 times with HBSS at 1500 rpm. for 5 min. The contaminated red blood cells in the cell pellets were lysed with lysing solution (see, Appendix II) and washed twice with HBSS. After the last wash, the cell pellets were resuspened in TC 199 medium (see, Appendix II) and adjusted to $4x10^8$ cells/ml. with TC 199 medium. Heat inactivated cell-free pleural fluid supernatants were diluted by a serial two-fold dilution with TC 199 medium. 200 Ul. of the leukocyte suspension were mixed in equal volumes with the undiluted or the serially two fold diluted pleural fluids or TC 199 medium as control and incubated at 37°C 5% CO_2 for 2 hours with mixing every 30 min. After preincubation, 10 Ul. of the pleural fluid-PMN cell mixtures or controls were placed in a well of TC 199 agarose medium (see, Appendix II) using triplicate wells and incubated at 37°C 5% CO_2 for 18 hours. The plates were then fixed by fixing agent (see, Appendix II) to determine the migration area as

decribed above. The migration index (M.I.) was expressed by the following formula.

$$\text{Migration Index} = \frac{\text{Migration area of PMN cells in the presence of pleural fluid}}{\text{Migration area of PMN cells in medium control}}$$

6. To determine the optimal PPD concentration for direct LIF test.

The optimal dose of PPD for LIF test is the minimum concentration of PPD that can stimulate enough LIF production from specifically sensitized lymphocytes to inhibit the leukocyte migration but is non-toxic to the cells. This primary study was performed in 2 Thai male patients with active pulmonary tuberculosis from Chulalongkorn Hospital. Both patients had abnormal chest X-rays, positive AFB in sputum smear were positive sputum culture for Mycobacterium tuberculosis and positive tuberculin skin test . Heparinized blood of these patients were obtained and purified polymorphonuclear cells were isolated by the method described above. Virgin cord blood leukocytes from normal newborn were studied in parallel to determine the toxic effect of PPD on the migratory activity of the migratory cells. The cell suspension at 2.4×10^8 cells/ml. was incubated with various concentrations of preservative-free PPD preparation at 37 C for 30 mins. After this

short incubation, these cell suspensions were allowed to migrate in the TC 199 agarose medium to perform the PPD-stimulated direct LIF test by the method described above. As shown in Figure 8, PPD concentrations higher than 100 Ug/ml. could inhibit the migration of all kinds of leukocyte suspensions including the cord blood leukocytes. This indicated that mean PPD concentration of over 100 Ug/ml. was toxic to the migratory activity of the migratory cells. The PPD concentration range between 50 to 100 Ug/ml. could cause migration inhibition of patients' leukocytes. Such inhibition of leukocyte migration was demonstrated to be the result of LIF production from the antigen-stimulated sensitized lymphocytes, since lymphocyte-depleted PMN suspensions from the same patients could no longer produce LIF (Figure 8). On the other hand, these antigen concentrations did not interfere with the migratory activity of lymphocyte depleted purified polymorphonuclear cell of these tuberculous patients or of the cord blood leukocytes. The PPD concentration at 100 Ug/ml. was therefore chosen for use in the subsequent experiment of direct LIF test.

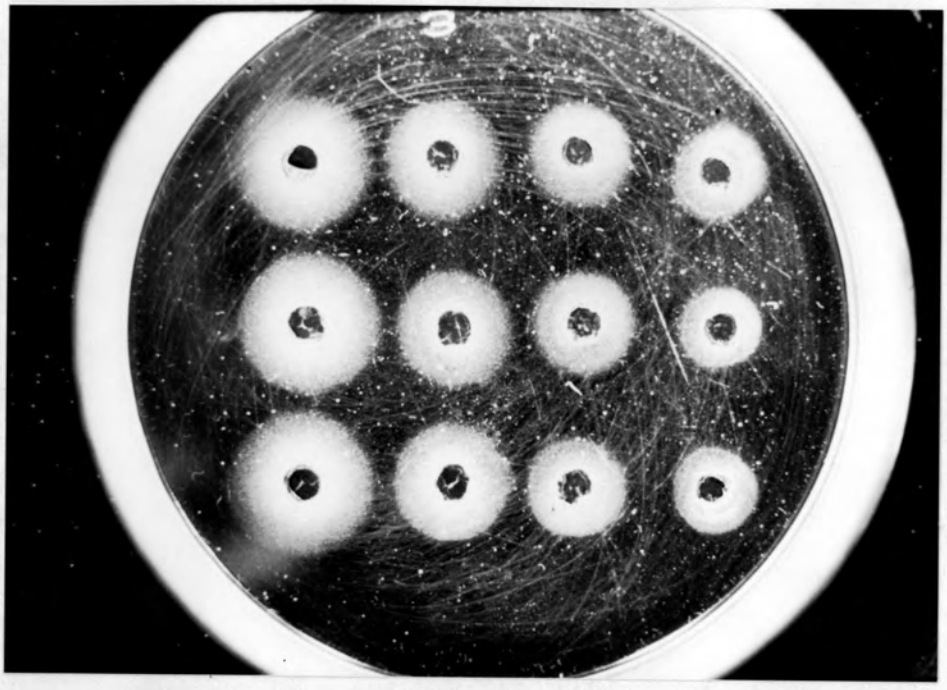

Figure 7. The migrating cells in LIF test.

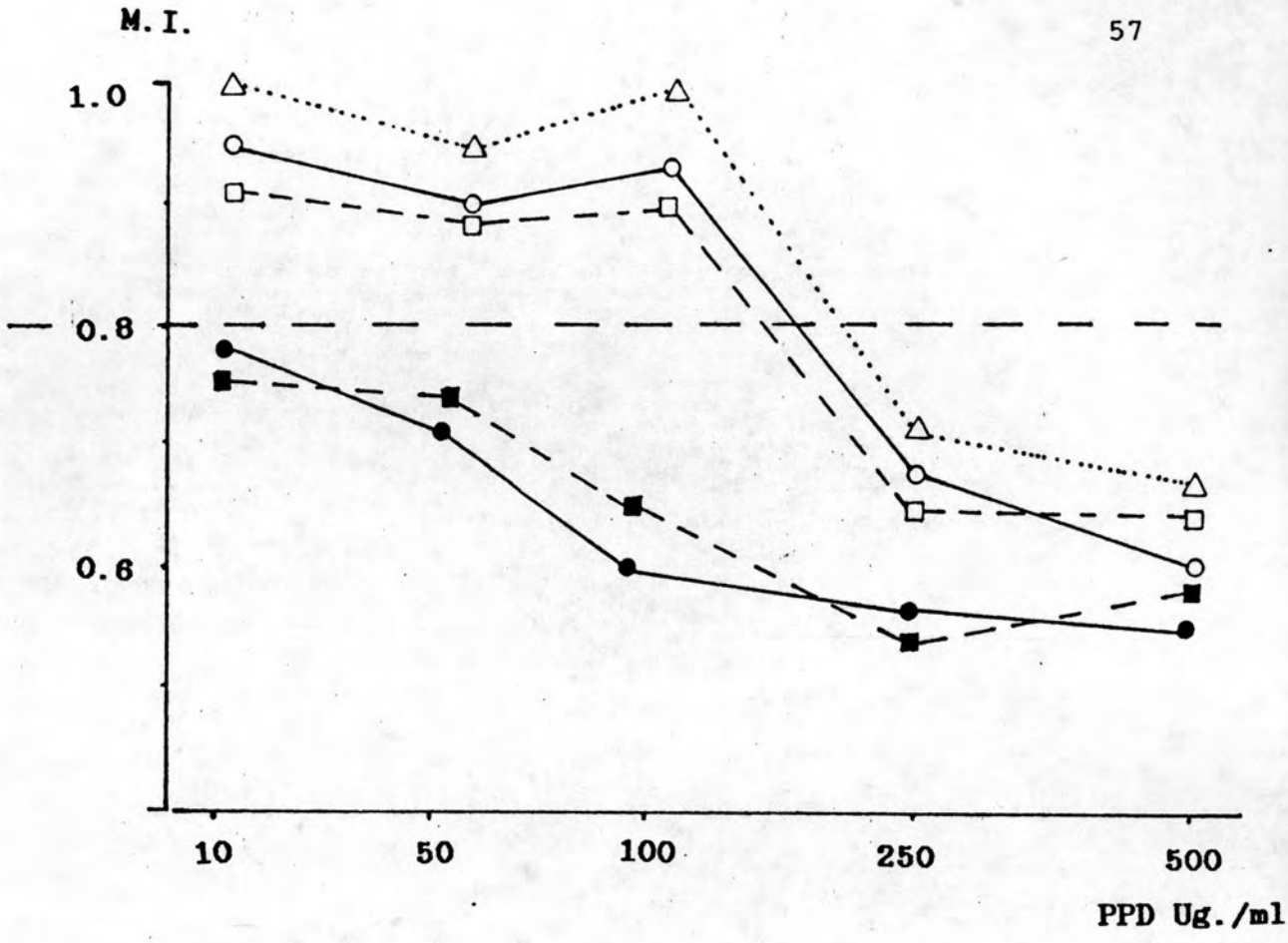

Figure 8. The effects of PPD concentrations on direct LIF test of various types of leukocyte suspension.

■- - - ■ Leukocytes of tuberculous patient No. 1

●——● Leukocytes of tuberculous patient No. 2

□- - - -□ Lymphocyte-depleted purified PMN of tuberculous patient No. 1

○——○ Lymphocyte-depleted purified PMN of tuberculous patient No. 2

△.....△ Cord blood leukocytes

Lymphocyte Transformation Test

1. In vitro lymphocyte transformation test.

Proliferative response of PFMC and PBMC to mycobacterial antigen. (i.e., purified protein derivative; PPD) and to mitogen (i.e., phytohemagglutinin ; PHA) was done in a 5 day microculture system according to the method of Oppenheim and Schecter (56) with slight modification. The PFMC and PBMC preperations as described were resuspended in tissue culture medium (TCM) containing RPMI 1640 supplemented with 5% heat inactivated pooled normal human AB serum, 100 U/ml. penicillin, 100 Ug/ml. streptomycin and 10 mM HEPES (see, Appendix II). and adjusted to a cell concentration of $2x10^6$ cells/ml. $2x10^6$ cells in 0.1 ml. volume were cultured in sterile flat bottom plastic microtiter plate (Nunc., Intermed, Roskilde, Denmark). PPD (Staten seruminstitut, Copenhagen, Denmark) or PHA (Gibco, Grand Island, N.Y., USA.) at 0.1 ml. volume in TCM was added to cell culture to give the final concentration of 1,10 Ug/ml. and 25 Ug/ml. respectivly for stimulated culture and 0.1 ml. TCM was used as the unstimulated or background control. The culture was done in triplicate and cultured at 37 C with 5% CO_2 for 5 days. On day 5, the cell cultures were pulsed with 20 Ul. of 25 UCi/ml. of tritiated thymidine (specific activity 6.7 mCi/mMol.; Amersham, UK.) to give

a final concentration of 0.5 UCi per well. After 18 hours incubation, the tissue culture plates were harvested on micro glass fiber filter paper (Whatman, N.J., USA.) with the automatic microcell harvester (Model CH 103, Dynatech Laboratory, USA.) with 2-3 cycles of automatic washing. The air-dried glass fiber filter paper of each well was dipped in 5 ml. of scintillation fluid (see, Appendix II) in the scintillation vial (Kimbel, Illinois, USA.) for β-ray counting, in a β-counter (model LSC-100C, Beckman,USA.). The average counts per minute (cpm.) of the triplicates both in the absence and in the presence of PPD and PHA control were used to calculate the △ cpm. and the stimulation index (S.I.) by the following formula :

△ cpm. = Average cpm. of stimulated culture - Average cpm. of unstimulated culture

$$\text{Stimulation Index (S.I.)} = \frac{\text{Average cpm. of stimulated culture}}{\text{average cpm. of unstimulated culture}}$$

2. Antigen preparation for in vitro stimulation test.

1000 Ug/ml. of purified protein derivative (PPD) RT 23 strain containing 0.01% chinosol as preservative (Statens seruminstitut, Copenhagens, Denmark.) was dialysed against several changes of in sterile phosphate buffer saline (PBS; see, Appendix II) pH 7.4 for 24 hours. This

preservative free PPD was aliquoted in sterile vials and stored in the dark at -70 C.

3. Dose response curve of PPD stimulation in lymphocyte transformation test.

To determine the optimal concentration of PPD to be used in the lymphocyte tranformation test, various concentrations of preservative-free PPD were tested with the lymphocytes from 2 patients with bacteriologically proved active pulmomary tuberculosis following the methods described above. The final concentrations of PPD in the culture system were 0.1, 1, 5, 10, 25 and 100 Ug/ml. AS shown in Figure 9, 1 to 10 Ug/ml. of PPD gave the highest $\triangle$ cpm. Therefore, 1 and 10 Ug/ml. of PPD were chosen as the optimal doses to stimulate lymphocyte proliferation in vitro in all of the experiments reported in this study.

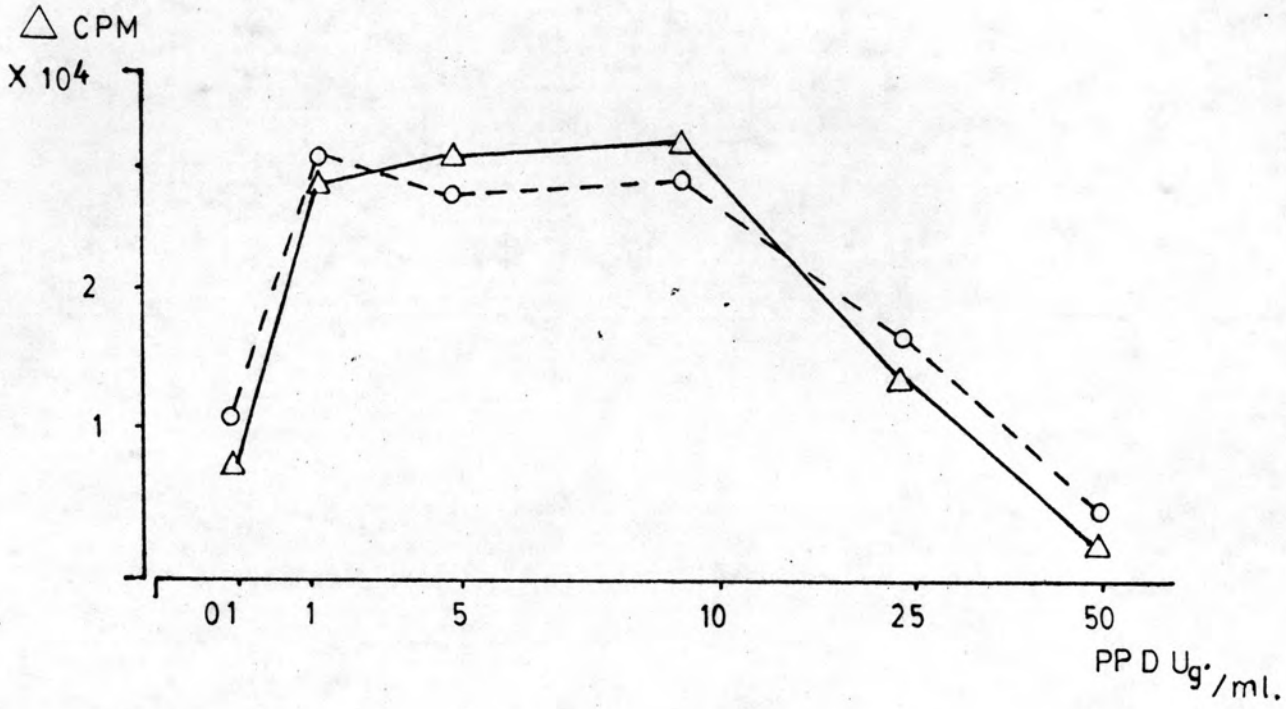

Figure 9. Dose response curve of PPD-stimulated proliferation of lymphocytes from tuberculous patients No. 1 (O- - -O) and No. 2 (△——△).