III. MATERIALS AND METHODS

A. Subjects:

1. HIV-negative donors: The normal control group included 22 HIV-negative donors (6 males and 16 females), with a mean age of 24.48 years (range 20-37 years). All the HIV-negative donors were in a good health at the time of blood collection. HIV-negative donors were used as normal control subjects for studying lymphocyte function and inhibition in plasma.

2. Patients: The patient groups consisted of 15 asymptomatic HIV-infected individuals (5 males and 10 females), with a mean age of 31.15 years (range 26-37 years). Fiftheen AIDS patients without *P. marneffei* infection (11 males and 4 females), with a mean age of 30.67 years (range 18-40 years), and 16 AIDS patients with *P. marneffei* infection (10 males and 6 females), with a mean age of 30.30 years (range 20-43 years). All HIV- infected patients were first diagnosed at Maharaj Nakorn Chiang Mai Hospital Faculty of Medicine. The diagnosis of HIV infection was made by both enzyme-linked immunosorbent assay (Vironostika HIV MIXT, Organon Teknika B.V., Boxtel, Holland) and particle-agglutination test (Serodia-HIV, Fujirebio Inc, Tokyo). The diagnosis of *P. marneffei* infection was made by isolation of *P. marneffei* from clinical specimens such as blood, bone marrow. Secondary identification of yeast cells characteristic in Wright's stained bone marrow aspirated and touch smears of skin specimens obtained by biopsy or in tissue sections stained

with Grocott-Gomori methenamine-silver nitrate (GGMS) or periodic acid Schiff (PAS).

B. Blood collection and mononuclear leukocyte preparation

Ten millilitres of venous blood was drawn into sterilized test tubes containing 500 units of preservative-free heparin (Novo Industrial, Copenhagen, Denmark). The mononuclear cells were obtained by Ficoll-Hypaque gradient centrifugation (Böyum, 1968). Briefly, the heparinized blood was diluted 1:1 with PBS, pH 7.2 following with upper laying on top of Ficoll - Hypaque solution (Phamacia, Sweden; Winthrop Laboratories; New York, USA) in sterile centrifuge tubes. The tube was centrifuged at 400 g for 30 minutes at room temperature. The upper layer containing plasma and platelets was removed and the PBMC band (at the interface) was collected and washed twice with sterile PBS and RPMI-1640 respectively. The PBMCs were then counted and adjusted to 1.5 x 10^6 cells/ml with complete RPMI-1640 (CRPMI) (see appendix 4). The viability of the final preparation should be greater than 97%, as determined by trypan blue exclusion test.

C. Preparation of crude sonicated *P. marneffei* antigen

Yeast form of *P. marneffei* strain 391 H isolated from hemoculture was subcultured on brain heart infusion (BHI) agar slants (see appendix 6) at 37 °C for 4-5 days. Yeast cells were harvested by washing with 5 ml of sterilized NSS per slant and the yeast cell suspension was individually inoculated into 50 ml of BHI broth in 250 ml erlenmeyer

flask. The cultures were then incubated at 37 °C with continuous shaking at 150 rpm for 3 days. On the day after, yeast cells were harvested by centrifugation at room temperature at 4,000 rpm for 10 minutes. The supernatant was removed, and yeast cells were collected and washed three times with sterilized NSS. Cell were resuspended with 5 ml of sterilized NSS and disrupted by the ultrasonicator (200 watts 30 seconds) for six times on ice-bath. The homogenate was centrifuged at 4,000 rpm for 15 minutes and the supernatant was collected and diluted in sterilized NSS at equal volume. The final step was to sterilize by passing through a 0.45 micron poresize of millipore membrane filter. The protein concentration was determined by Lowry's method.

D. Purification procedure

Molecular-mass profiles were estimated by separating the proteins of *P. marneffei* with Sephadex G-100 gel filtration. Two millilitres. of samples were collected with PBS pH 7.4 from a 53 length x 1.5 cm-diameter column in 3 ml fraction at a flow rate 3 ml/10 min and measured for protein at OD 280 nm. The fractions were pooled and dialyzed in PBS at 4 °C for 24 hours, and concentrated by lyophilization method. The total protein was determined by Lowry's method.

E. *In vitro* lymphocyte transformation test

1. Determination of the suboptimal and optimal concentration of PHA-P

One hundred microlitres of 1.5 x 10^6 PBMCs/ml were added to a well of microculture plate (NUNCLON, DELTA) containing RPMI-1640 medium that supplemented with antibiotic, 12.5 mM HEPES buffer, 5 x 10^{-5} M 2-mercaptoethanol (2-ME), 1 mM sodium pyruvate and 10% heat-inactivated FCS and various concentrations of PHA-P (0, 0.0156, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 µg/ml; Wellcome) in the same final volumes. The culture plates were incubated at 37 °C in 5% CO_2 in air at 100% humidity for 72 hours. Eighteen hours before harvesting the cells were pulsed with tritiated-thymidine 0.2 µCi/well (47 Ci/mmol; Amersham). The cultured cells were harvested with a semi-automatic cells harvester onto the glass microfibre filters. The filters were dried at room temperature overnight then put in the scintillant (see appendix) and then measured for the radioactivity by a liquid scintillation counter (LKB, WALLAC). Each determination was performed in triplicate, and the radioactivity was expressed in term of cpm.

2. Determination of the optimal concentration of PBMC

One hundred microlitres of various concentrations of PBMC (0.25 x 10^6, 0.5 x 10^6, 1.0 x 10^6, 1.5 x 10^6, 2.0 x 10^6 and 4.0 x 10^6 cells/ml) were placed to the wells of microculture plate containing RPMI-1640 medium

that supplemented with 10% heat-inactivated FCS and 2 µl.of PHA-P (1.0 µg/ml) (the optimal concentration of PHA-P). The plate was cultured at 37 °C in 5% CO_2 in air at 100% humidity for 72 hours and processed as described above.

3. Determination of the optimal concentration of PHA-P and PBMC by block titration method

One hundred microlitres of various concentrations of PBMC (0.5 x 10^6, 1.0 x 10^6 and 1.5 x 10^6 cells/ml) were placed to the wells of microculture plate containing RPMI-1640 that supplemented with antibiotic, 12.5 mM HEPES buffer, $5x10^{-5}$ M 2-mercaptoethanol (2-ME), 1 mM sodium pyruvate and 10% FCS and various concentration of PHA-P (0.25, 1.0 and 20 µg/ml). The culture plates were incubated at 37 °C in 5% CO_2 in air at 100% humidity for 72 hours and processed as described above.

4. Determination of the suboptimal and optimal concentration of crude sonicated *P. marneffei* antigen

One hundred microlitres of 1.5 x 10^6 PBMCs/ml were placed to a well of microculture plate containing RPMI-1640 medium that supplemented with antibiotic, 12.5 mM HEPES buffer, $5x10^{-5}$ M 2-mercaptoethanol (2-ME), 1mM sodium pyruvate and 10% heat-inactivated FCS and various concentrations of crude sonicated *P. marneffei* antigen (0, 0.32, 0.64, 1.28, 2.56 and 5.12 µg/well) in the same final volumes. Then the plates were cultured for 7 days and processed as described above.

5. Determination of the suboptimal and optimal concentration of PPD

One hundred microlitres of 1.5 x 10^6 PBMCs/ml were placed to a well of microculture plate containing RPMI-1640 medium that supplemented with antibiotic,12.5 mM HEPES buffer, $5x10^{-5}$ M 2-mercaptoethanol (2-ME), 1mM sodium pyruvate and 10% heat-inactivated FCS and various concentrations of PPD (0, 0.3125, 0.625, 1.25, 5.0, 10.0 and 20.0 μg/ml) in the same final volumes. Then the plates were cultured for 7 days and processed as described above.

6. Determination of the suboptimal and optimal concentration of the fraction *P. marneffei* antigen after gel filtration

One hundred microlitres of 1.5 x 10^6 PBMCs/ml were added to a well of microculture plate containing 10% of heat-inactivated fetal bovine serum and various concentration of two distinct protein peaks of *P. marneffei* antigen (0, 0.16, 0.64, 1.28, 2.56, 5.12 and 8.00 μg/well) in the same final volumes. Then the plates were culture for 7 days and processed as described above. The radioactivity in stimulated with each fraction of *P. marneffei* antigen was compared to crude sonicated antigen in the similar concentration.

7. Determination of the ability of lymphocytes transformation in AIDS patients stimulated with PHA- P

PBMCs were prepared from the sera of HIV-negative donors, asymptomatic HIV-infected individuals, and AIDS patients without or with *P. marneffei* infection. One hundred microlitres of 1.5 x 10^6 PBMCs/ml from each donor was placed to a well of a microculture plate containing RPMI-1640 medium that was supplemented with antibiotic, 12.5 mM HEPES buffer, 5 x 10^{-5}M 2-mercaptoethanol (2-ME), 1 mM sodium pyruvate, 10% heat-inactivated FCS and which contained the suboptimal or optimal concentration of PHA-P in the same final volumes. Then the plates were cultured for 3 days and processed as described above.

8. Determination of the ability of lymphocyte transformation in AIDS patients stimulated with crude sonicated *P. marneffei* antigen

PBMCs were prepared from HIV-negative donors, asymptomatic HIV-infected individuals and AIDS patients without or with *P. marneffei* infection. One hundred microlitres of 1.5 x 10^6 PBMCs/ml form each donor were placed to a well of a microculture plate containing RPMI-1640 medium that was supplemented with antibiotic, 12.5 mM HEPES buffer, 5 x 10^{-5} M 2-mercaptoethanol (2-ME), 1 mM sodium pyruvate, 10% heat-inactivated FCS and which contained the suboptimal or optimal concentration of crude sonicated *P. marneffei* in the same final volumes. Then the plates were cultured for 7 days and processed as described above.

9. Determination of the ability of lymphocyte transformation in AIDS patients stimulated with PPD

PBMCs were prepared from HIV-negative donors, asymptomatic HIV-infected individuals and AIDS patients without or with *P. marneffei* infection. One hundred microlitres of 1.5 x 10^6 PBMCs/ml form each donor were placed to a well of a microculture plate containing RPMI-1640 medium that was supplemented with antibiotic, 12.5 mM HEPES buffer, 5 x 10^{-5} M 2-mercaptoethanol (2-ME), 1 mM sodium pyruvate, 10% heat-inactivated FCS and which contained the suboptimal or optimal concentration of PPD in the same final volumes. Then the plates were cultured for 7 days and processed as described above.

10. Identification of the active fraction of *P. marneffei* antigen after gel filtration

One hundred microlitres of 1.5 x 10^6 PBMCs/ml were added to a well of microculture plate containing 10% of heat-inactivated fetal bovine serum and various concentration of two distinct protein peaks of *P. marneffei* antigen (0, 0.16, ,1.28, and 5.12 µg/well) in the same final volumes. Then the plates were culture for 7 days and processed as described above. The radioactivity in stimulated with each fraction of *P. marneffei* antigen was compared to crude sonicated antigen in the similar concentration.

11. Determination of plasma form AIDS patient with *P. marneffei* infection inhibit proliferation of normal T cell

One hundred microlitres of 1.5 x 10^6 PBMCs/ml from HIV-negative donors were placed in a well of a microculture plate containing RPMI-1640 medium that was supplemented with 10% autologous plasma or asymptomatic HIV-infected individuals plasma or AIDS patient without or with *P. marneffei* infection plasma. Then the PBMC were stimulated with suboptimal or optimal concentration of PHA-P. The plates ware cultured at 37 °C in 5% CO_2 in air at 100% humidity for 72 hours. Eighteen hours before harvesting the cells were pulsed with tritiated-thymidine 0.2 μCi/well. The cultured cells were harvested with a semi-automatic cell harvester onto the glass microfibre filters. The filters were dried at room temperature overnight then put in the scintillant and measured for the radioactivity by a liquid scintillation counter. Each determination was performed in triplicate, and the radioactivity was expressed in term of cpm. The amount of tritiated-thymidine incorporation by normal cell culture with patient plasma compared to the amount of tritiated thymidine incorporation by normal cell culture with autologous plasma indicated the ability of patient plasma to inhibit T cell proliferation.

12. Determination of the concentration of plasma from AIDS patients to inhibit proliferation of normal T cell

One hundred microlitres of 1.5 x10^6 PBMCs/ml from HIV-negative donor were placed in a well of a microculture plate containing RPMI-1640 medium that was supplemented with 5%, 10%, 20% and 30% of autologous plasma, homologous plasma, asymptomatic HIV-infected individual plasma or AIDS patient without or with *P. marneffei* infection plasma. Then the PBMC were stimulated with suboptimal and optimal concentration of PHA-P. The plates were incubated for 3 days and processed as described above. The amount of tritiated-thymidine incorporation by normal cell culture with patient plasma was compared to the amount of tritiated-thymidine incorporation by normal cell culture with autologous plasma in the same concentration of plasma indicate the ability of patient plasma to inhibit T cell proliferation.