V. DISCUSSION

The specific immune responses against a microorganism are classified into two types, based on the components of the immune system that mediate the response (Abbas et al., 1994). The first is mediated by B lymphocytes in the blood system. This response provides for specific recognition and elimination of antigens by synthesis and secretion of antibodies, and is called humoral mediated immunity. The other type of response cell mediated immunity. This is mediated by T lymphocytes. Cell mediated immunity can be transferred to unimmunized individual with cells from an immunized individual but not with plasma or serum. CMI responses recognize a variety antigens from bacteria, viruses, fungi and parasites. Cell mediated immune reactions may also be important for elimination of cells that express foreign MHC molecules, as in an allograft, or express tumor-specific antigens, as in a malignant tumor (Davis et al., 1990). The different types of cell-mediated immune reactions may result from T cell recognition of antigen. Penicilliosis marneffei is the infectious disease caused by the fungus, *P. marneffei.* The clearance of *P. marneffei* depends on cell mediated immunity rather than humoral immunity.

In the northern Thailand, this mycosis was the third most common opportunistic infection in AIDS patients after tuberculosis and cryptococcosis. It was found that the yeast form of *P. marneffei* could be isolated from nasal swab of AIDS patient infected with this fungus (Vithayasai, 1994). This might be the reason that those certain persons

may get infected from *P. marneffei* by airborne (Deng et al., 1986). Some still remain healthy because of their own *P. marneffei* specific immune response. In contrast, there is the exception in HIV-infected patients, the *P. marneffei* specific memory T cells are destroyed by HIV virus or viral products. Therefore, the HIV-infected patients showed symptoms when they were reactivated with this fungus.

The purpose of this study is, firstly, to evaluate the CMI response to crude sonicated *P. marneffei* antigen in asymptomatic HIV-infected individuals, AIDS patients without and with *P. marneffei* infection compare to HIV-negative donors. Secondly, to determine whether plasma from those cohorts might have an inhibitory effect on the CMI response.

In this study, peripheral mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient centrifugation (Böyum, 1968). The purity of PBMC from normal donor, asymptomatic HIV-infected individuals, and AIDS patients without and with *P. marneffei* infection were more than 95%. These results indicated that this method yielded relatively pure PBMC. The viability of these cells were evaluated by trypan blue dye exclusion method. It was found that the viability of PBMC from each group of the patients and all HIV-negative donors was more than 97%.

Cell mediated immunity of each group of the patients and HIV-negative blood donors was studied by the lymphocyte transformation test. The lymphocytes were stimulated with PHA-P which mainly stimulates T-cells. The mechanism of action is to initiated the reaction specifically with various sugars of glycoprotein on the lymphocyte surface and also induce differentiation of mature cells into blast cells (Davis et al.,1990). In this

experiment, PHA-P was used at a suboptimal and the optimal concentration of 0.125 μg/ml, 1.0 μg/ml, respectively. The suboptimal and optimal concentration were determined from the titration curve. The different concentrations of PHA-P were due to the number of mononuclear cell per culture which was 1.5 x 10^5 cells/well. The incorporation of tritiated-thymidine reflected lymphocyte transformation and was measured by the liquid scintillation counter. In an *in vitro* specific antigen induced proliferation assay of the PBMC, crude sonicated *P. marneffei* protein and PPD were used as antigens. The number of PBMC used in this experiment was 1.5 x 10^5 cells/well and 7 days cultured with 0.32 and 2.56 μg/well of crude sonicated *P. marneffei* antigen or 0.3125 and 10.0 μg/ml of PPD which were used as suboptimal and optimal concentration, respectively. Subsequently, 0.2 μCi of tritiated-thymidine was added to each well at the 18 hours before harvesting.

The study was conducted to the lymphocyte transformation by both suboptimal and optimal concentration of PHA-P stimulation in asymptomatic HIV-infected individuals, AIDS patients without and with *P. marneffei* infection. The result showed that there was a significant decreasing when compared to HIV-negative donors ($p < 0.001$). Moreover, the lymphocyte transformation compared among each group of patients, revealed that in suboptimal concentration, the PBMC from asymptomatic HIV-infected individuals exhibited significant higher response than AIDS patients without and with *P. marneffei* infection ($p < 0.025$, and < 0.01, respectively). Meanwhile, in optimal concentration, it was no significant higher response than AIDS patients without *P. marneffei*

infection ($p > 0.1$), but it was significant higher response than AIDS patients with *P. marneffei* infection ($p < 0.05$). In addition, the both suboptimal and optimal concentration of PHA-P induced lymphocyte transformation in AIDS patients without *P. marneffei* infection was significant higher response than AIDS patients with *P. marneffei* infection. ($p < 0.05$).

Furthermore, the PBMC derived from asymptomatic HIV-infected individuals, AIDS patients without and with *P. marneffei* infection were stimulated with the suboptimal and optimal concentration of crude sonicated *P. marneffei* antigen. It was found that the lymphocyte transformation in asymptomatic HIV-infected individuals and also in both groups of AIDS patients decreased significantly when compared to the HIV-negative donors ($p < 0.001$). The lymphocyte transformation was compared in each group of patients and it was found that in suboptimal concentration, the PBMC from asymptomatic HIV-infected individuals exhibited significant higher response than AIDS patients without and with *P. marneffei* infection ($p < 0.025$). Meanwhile, it was not in optimal concentration ($p > 0.1$). Moreover, the lymphocyte transformation in AIDS patients without *P. marneffei* infection was no significantly different response when compared to AIDS patients with *P. marneffei* infection in both suboptimal and optimal concentration ($p > 0.1$, and > 0.05, respectively).

PBMC derived from such those samples were also studied with suboptimal and optimal concentration of PPD. It was found that the lymphocyte transformation in asymptomatic HIV infected individuals,

AIDS patients without and with *P. marneffei* infection decreased significantly when compared to the HIV negative donors ($P < 0.001$). Whereas, lymphocyte transformation in both suboptimal and optimal concentration of PPD compared in each group of patients, showed that PBMC from asymptomatic HIV-infected individuals exhibited no significant higher response than AIDS patients without and with *P. marneffei* infection ($p > 0.1$), but it was the exception in AIDS patients without *P. marneffei* infection when their PBMC stimulated with the optimal concentration ($p < 0.001$). Furthermore, the both suboptimal and optimal concentration of PPD induced lymphocyte transformation in AIDS patients without *P. marneffei* infection was no significantly different response when compared to AIDS patients with *P. marneffei* infection ($p >0.1$).

A diminished response of lymphocyte transformation by PHA-P stimulation was found in AIDS patients without and with *P. marneffei* infections. Moreover, the lymphoproliferative response in asymptomatic HIV-infected individuals could be divided into two groups, the former group was high and the latter group was low. The decrement of lymphocyte transformation response to PHA-P in asymptomatic HIV-infected individuals correlated to the absolute counts of $CD4^+$ T cell and the AIDS development.

When the PBMCs were stimulated with crude sonicated *P. marneffei* antigen, the lymphoproliferative response in HIV-negative donors could be classified in to two groups. Firstly, the major population was high response and the minor population was a low response. In contrast, in

asymptomatic HIV-infected individuals, the major population of patients showed a low response. Furthermore, the lymphoproliferative response in all AIDS patients without and with *P. marneffei* infection were not found. It was indicated that, both HIV-negative and HIV positive persons had been infected with *P. marneffei*. In HIV-negative persons still remained healthy because of their own *P. marneffei* specific immune response. This was the exception in HIV-infected patients, the memory T cells to *P. marneffei* were destroyed by HIV virus or viral products so they showed symptoms when they were reactivated with this fungus.

When PBMC was stimulated with PPD, the high lymphoproliferative response was also found in the major population of HIV-negative donors. In contrast, in asymptomatic HIV-infected individals, the high response was found in the minor population. Moreover, the lymphoproliferative response in AIDS patients without and with *P. marneffei* infection were not found. This result indicated that the PPD specific memory T cells in HIV-infected patients were destroyed by HIV virus or viral products. This leads to a high risk for disease with *M. tuberculosis*.

This data are consistent with studies previously reported. In 1987 and 1989, Hofmann and his colleagues demonstrated that responses to PHA were reduced only in clinically ill HIV seropositive patients. In contrast, the response to PWM, recall antigens (*C. albicans* antigen, cytomegalovirus antigen) were profoundly reduced in most HIV seropositive subjects including the asymptomatic group. Furthermore, the decrease in lymphocyte transformation responses to PWM and recall

antigen correlated to the clinical condition and the absolute counts of $CD4^+$ T cell (Hofmann et al., 1987; Hofmann et al., 1989). In asymptomatic HIV infected individuals, loss of both $CD4^+$ and $CD8^+$ T cell function was observed. Initialy, T cell responses to recall antigen were lost. T cells were not able to respond to antigen presented in the context of self-MHC (Clerici et al., 1991). Subsequently, the responses to alloantigens and mitogens were lost (Via et al., 1990). Several groups have reported individuals in whom only recall antigen responses were affected. Such a defect could at least partially be explained by the quantitative loss of memory cells (De Martini et al.,1988; De Paoli et al, 1988 and Van Noesel et al.,1990). Schnittman and his colleagues used enriched subsets of T cells to demonstrate that CD45R0 expressing $CD4^+$ T cells were preferentially infected *in vitro* by HIV and that $CD45R0^+$ T cells constitute the major population of HIV-infected cells in the peripheral blood *in vivo*. It was proposed that by preferential viral infection, HIV induces more profound deficiency in the memory compartment of $CD4^+$ T cells. Moreover, they proposed that $CD45R0^+$ T cells from HIV-infected individuals were unresponsive to recall antigen but were still able to respond to mitogens. These datas could be interpreted as a preferential loss of MHC-restricted responses rather than memory cell function (Schnittman et al.,1990). The mechanism of inducing in general T cell defects during HIV infection, was the low frequency of infected cells that cause direct viral infection, an unlike explanation for T cell dysfunction (Schnittman et al.,1990). Several mechanisms have been proposed as a systemic explanation for T cell dysfunction in HIV infection. Chirmule

and his colleagues reported interaction of HIV envelope protein gp120 with $CD4^+$ results in suppressed tetanus toxoid antigen stimulated $CD4^+$ T cells proliferation. The mechanism of immune suppression by gp120 was interference with the initiation of signal transduction through the T-cell receptor complex (Chirmule et al., 1990). In addition, overproduction of the immunosuppressive cytokines TGF-β (Kekow et al., 1990) and IL-10 (Clerici et al.,1993) has been associated with decreasing in T cell function. T cell dysfunction might be the result of defects in antigen presentation due to infection of the accessory cells (Meyaard et al., 1993). T cells from asymptomatic HIV-infected individuals resemble anergic cells as defined in cell culture models. Anergy might be induced by lack of correct accessory molecules or in appropriate cytokine secretion by antigen presenting cells. Indeed, impaired IL-12 was produced by monocytes from asymptomatic HIV-infected individuals (Chehimi et al., 1994). However the mechanism of anergy in HIV-infected patients that cause defect in lymphocytes was not fully understood.

Moreover, in this experiment, crude sonicated *P. marneffei* protein purified by Sephadex G-100 gel filtration collumn provided two distinct protein peaks eluted from the column. A molecular weight (MW) of protein antigen in peak 1 was more than 100 kD, incontrast, peak 2 was less than 100 kD. Furthermore, protein antigen activity was found only in peak 1. This was indicated that the peak 1 protien antigen, molecular weight more than 100 kD had more specific antigenic determinant to memmory T cells than peak 2 protein antigen, molecular weight of less than 100 kD.

Another cause of defective lymphocyte transformation may be the presence of an inhibitory factor in the plasma. We have also demonstrated that lymphocyte transformation of HIV-negative donors decreased after incubation with the plasma from asymptomatic HIV infected individuals, and with the plasma from AIDS patient without and with *P. marneffei* infection. Lymphocytes from HIV-negative donors stimulated with suboptimal and optimal concentration of PHA-P in the presence of plasma from AIDS patient without and with *P. marneffei* infection proliferated significantly less than lymphocytes stimulated with plasma from asymptomatic HIV-infected individuals ($p < 0.001$). On the other hand, there was no significant differencein in lymphoproliferative response assay between AIDS patients without and with *P. marneffei* infection in both suboptimal and optimal concentration of PHA-P ($p > 0.2$). Furthermore, it was found that the percent inhibition was correlated to the concentration of patient plasma.

This result is correlated with to previous studies. Siegel and his colleagues demonstrated that serum from AIDS patients suppressed PHA-P induced IL-2 production (Siegel et al., 1985). However, PHA induced proliferation of normal lymphocytes in the presence of AIDS patient serum was not significantly lower than normal serum. This may be explained by the use of a high concentration of PHA (5 μg/ml). Moreover, Siegel and his colleagues demonstrated that the suppressive effect to IL-2 production in AIDS patient serum was not mediated by interferon, cortisol, immunoglobulin G or M, or immune complexes, because the suppressive activity was not lost after incubation at pH 3 or 10 for 6 hours followed by

neutralization. The activity persisted through heating to 60 °C for 6 hours but 75% of activity was lost during heating to 100 °C for 5 minute Suppressive activity persisted after three cycles of freezing and thawing and it was not ether extractable (Siegel et al., 1985).

It was suggested that the inhibitory factors might be gp 120, the Nef protein of HIV or p29. Mann and his colleagues reported that the gp120 viral glycoprotein suppressed immune function by binding to the CD4 molecule, which turns off the signal for lymphocytes to respond to PHA stimulation (Mann et al, 1987). Collet and his colleagues suggested that the HIV nef gene product down regulated IL-2 production by $CD4^{+}$ T cells that were also stimulated with PHA (Collett et al., 1996). The inhibitory factor has also postulated to be p29, the inhibitory factor produced by HIV-infected adherent cells. The p29 suppressed PHA induced proliferation of normal T cells by decreasing the expression of high affinity IL-2 receptors and production of IL-2 (Ammar,1992). Furthermore, Drouhet and his colleagues demonstrated that fungal antigens, such as mannan of *C. albicans* and glucuronoxylomannan of *C. neoformans,* were capable of suppressing cellular immunity (Drouhet, 1983). Therefore, these fungi may play a role as a cofactor in the development of AIDS. In addition mannan in candidosis, and glucuronoxylomannan in cryptococcosis were the most frequently observed in blood circulation of AIDS patients with fungal diseases. *In vivo*, the antigen specific T cell mediated suppression corresponds with disease since it disappears in patients treated with an appropriate effective therapy of ketoconazole (Drouhet, 1983). *P. marneffei* antigen (mannan or/and

glucuronoxylomannan) may be an immunosuppressor of cellular immunity as there was also report for the antigens of *C. albicans* and *C. neoformans*. However in this study we found that normal lymphocytes stimulated in the presence of plasma obtained from AIDS patient infected with *P. marneffei*, was not significantly lower than that stimulated in the presence of plasma obtained from AIDS patient without *P. marneffei* infection.

In conclusion, the present study demonstrated that decreasing in response to PHA-P of *in vitro* lymphocyte transformation, and decreasing in response to crude sonicated *P. marneffei* antigen by peripheral blood mononuclear cells from asymptomatic HIV-infected individuals, and AIDS patients without and with *P. marneffei* infection. Mononuclear cells derived from asymptomatic HIV infected individuals and both group of AIDS patients were significant decreased in response to PHA-P and *P. marneffei* antigen than HIV-negative donors. These abnormalities might play a major role in susceptibility to systemic *P. marneffei* infection in HIV-infected patients. Presence of inhibitory activity on *in vitro* lymphocyte transformation (plasma from asymptomatic HIV-infected individuals and both groups of AIDS patients) also featured an immunosuppression in HIV infection.

Further studies, to prevent the cross reaction between *P. marneffei* antigen to other fungi antigen, the *P. marneffei* antigen must be purified by using HPLC technique. With the adventage of purified *P. marneffei* antigen, it can be used to study the epidermology and follow up the CMI response, especially in asymptomatic HIV infected individuals by mean of

skin test. To evaluate the CMI response is valiable, if preliminary skin test is positive and eventually decline, drug treatment should then be performed in such patients. However, asymptomatic HIV infected individuals who give negative skin test, should isolated from patients suffering with *P. marneffei*, therefore, to decrease the risk of *P. marneffei* infection.

To analyze the nature of inhibitory factors in AIDS patient plasma. Therefore, it can be removed in order to improve of cell-mediated immunity. The experiments should be designed to separate this factor from other plasma components and test for CMI suppression. This experiments can be done by culturing normal lymphocytes with such factor and evaluating for the lymphocyte transformation, such as IL-2 production, MIF production, lymphocyte mediated cytotoxicity, and lymphotoxin production. The molecular weight of the inhibitory factor can be determined by sodium dodecyl sulfate polyacryamide gel electrophoresis (SDS-PAGE) and the biochemical nature of this factor can be determined by biochemical technique, such as enzyme activity.

These studies should provide new insights into the immunopathology of AIDS and may lead to techniques for blocking the production of the factor, removing or inactivating it, or blocking, bypassing, or countering its effects and may thus contribute to rational design of immunotherapy for AIDS.