V. DISCUSSION

A. Detection of immunogenic proteins in crude concentrated culture filtrate antigens

The previous study of immunogenic proteins in culture filtrate of *P. marneffei* yeast-form by Vanittanakom et al (1997) showed that four immunogenic proteins of 200, 88, 54 and 50 kDa were produced in large quantity during the deceleration and early stationary phase of growth. They were reacted with IgG in individual sera derived from 33 AIDS patients with penicilliosis in percentage of 72.7, 93.9, 60.6 and 57.6, respectively. The bands of 88, 54 and 50 kDa gave strongly reactions with half of the serum samples. Interestingly, one serum sample derived from an AIDS patient could be strongly reacted to the 54 and 50 kDa proteins two months before the definite diagnosis by fungal culture. These results indicated that 54 and 50 kDa antigens were relatively specific to the *P. marneffei* infection. However, the 200 and 88 kDa proteins elicited weak reactivity in high proportions both AIDS patients and normal, so they may be common antigens that occur in the other environmental fungi.

In this study protein and immunogen profiles of crude culture filtrate antigens of *P. marneffei* 391H and 302BM were confirmed by the same method as described in materials and methods. These fungi were grown on 500 ml BHI broth at $37^{\circ}C$ for 4-5 days in a 2 liters erlenmeyer flask shaker water bath with 140 rev min^{-1} (the culture turned brown-red pigment in color). The cultures were harvested and concentrated with 70% ammonium sulfate precipitation as described by Mekaprateep (1995) which almost proteins were reacted with patient's sera by immunoblot assay. The proteins were concentrated 50-fold in PBS from the initial culture volume. For each electrophoresis, the protein sample was approximately diluted (1:4) and 10 µl was applied per lane. The protein bands visualized on CBB-R250 stained gel were sharp with low background. The protein concentrations could be quantified according to the method of Bradford using a prepared reagents from BIO-RAD laboratories and bovine serum albumin (BSA) as the standard. Samples (10 µl) with appropriate dilutions were added with 200 µl of Bradford reagent and incubated for 15 min at RT. The resulting blue color was measured spectrophotometrically at 595 nm against blank

which prepared by using D.W. added with reagent. The amount of proteins from two crude antigens (391H and 302BM) were 3.7 and 3.0 mg/ml, respectively. The profiles of crude proteins from CBB R-250 stained gels and from immunoblots were similar in two isolates of *P. marneffei* and were similar to previous study by Vanittanakom et al (1997). Three bands of 88, 54 and 50 kDa protein were highly immunogen recognized by IgG antibodies in pooled sera from 10 AIDS patients infected with *P. marneffei* or anti-rabbit serum raised against crude concentrated proteins of *P. marneffei* yeast-form. Conversely, they did not react with antibodies from pooled sera of 10 healthy subjects or pre-immune rabbit serum except the band of 88 kDa antigen appeared weak positively reaction. The intensity of interesting proteins (88 and 50 kDa) among two-isolates of *P. marneffei* 391H and 302BM was different. The amount of 88 kDa protein was large secreted from *P. marneffei* 391H, whereas large amount of 50 kDa antigen was secreted from *P. marneffei* 302BM. Therefore, these two isolates of *P. marneffei* were used as subjects for purifying interesting proteins.

B. Partial purification of 88 kDa and 50 kDa protein antigens

Several studies have now identified a number of antigenic determinants in secreted antigen preparation from *P. marneffei* (Chongtrakool et al, 1997; Vanittanakom et al,1997). In addition, there had one report about purification of three *P. marneffei* antigens (61, 54 and 50 kDa) from cytoplasmic yeast antigens (CYA) which specifically recognized by IgG antibodies in penicilliosis patient's sera (Jeavons et al, 1998). Their experiments were achieved by using liquid isoelectric focusing combined with Prep-cell preparative gel electrophoresis methods. Two antigens (61 and 54 kDa) could be purified to homogeneity by preparative gel electrophoresis and the 50 kDa antigen was partially purified by the same technique. However, from the past to date there was no report involving purification and characterization of the antigens secreted from *P. marneffei*.

In this study, the interesting highly immunogenic proteins (88 and 50 kDa) were purified by using preparative gel electrophoresis and followed with two-dimensional gel electrophoresis technique. The Prep-cell Model 491 apparatus was consisted with discontinuous polyacrylamide gel (7.5% resolving gel, 4% stacking gel) as described in materials and methods. Three milliliters of crude proteins sample from *P. marneffei* 391H was mixed with an equal volume of 2X sample buffer and loaded

on stacking gel surface. Electrophoresis was carried out in cold room with a constant voltage of 250 V for 3-4 h until the bromphenol blue tracking dye ran out of the gel and the elution from gel electrophoresis was collected by using fraction collector with the flow rate of 2.5 ml/min. The volume of each fraction was 2.5 ml and about 250 fractions were collected. The proteins (88 and 50 kDa) which were eluted in elution buffer were analyzed by SDS-PAGE / Western blotting assay after concentrating the proteins with cold acetone precipitation. The 50kDa protein was continuously eluted in fractions of 50-70 with non-homogeneity. This antigen was difficult to purify since it was secreted in small amounts in crude proteins of *P. marneffei* 391H and its position on the gel was very closed to other proteins. This result was similar to the report by Jeavons et al (1998). In contrast, the 88 kDa antigen was continuously eluted as a single band from fractions of 160-210. It may be influenced this antigen presented in large amounts of the same crude proteins and its position was not so closed to other proteins. However, other lower molecular mass proteins contamination were seen after all fractions of 160-210 were pooled and concentrated by ultrafiltration and centricon device (MWCO 10 kDa). The contaminated proteins may be degraded form of this protein or these small proteins may be presented very few amounts in each fraction that could not be visualized on CBB-R250 stained gel in early detection. They might have been concentrated together with the 88 kDa protein and have reached to level that could be detected in secondary detection.

To purify 50 kDa protein antigen which presented large amounts in crude proteins of *P. marneffei* 302BM, the preparative gel electrophoresis using Mini-Protean II apparatus (BIO-RAD laboratories) with preparative comb (1.0 mm or 1.5 mm thickness) was conducted. The SDS-PAGE with 10% polyacrylamide gel (10%T 2.6%C) and 4% stacking gel was carried out with a constant voltage of 150 V for 1 hr. The volume of loading sample was about 150 and 200 µl, respectively. After staining gel with 0.1% CBB-R250, the band of interesting proteins (88 and/or 50 kDa) from about 20 gels were cut with a scalpel and pooled to get enough solution. The cut gels were minced with a 5 ml syringe and put into glass tube of electro-eluter apparatus (BIO-RAD). The procedure was done as described in the instruction manual. The eluted protein that retained on membrane cap (MW. cut off 12-15 kDa) was concentrated by cold acetone precipitating which described by Ortiz et al (1996).

The concentrated protein was analyzed again with SDS-PAGE and Western blotting assay. Its molecular weight was corrected and the antigenicity was retained comparing with crude concentrated proteins which run on the same gel. Although, the interesting proteins (88 and 50 kDa) could be isolated from crude proteins by following the two methods that described above, the yield of these partially purified proteins was not enough to determine their concentrations by Bradford assay.

C. Further purification of partially purified proteins (88 and 50 kDa)

Previous studies have demonstrated that two-dimensional polyacrylamide gel electrophoresis technique could resolved 1,100 different components from *Escherichia coli* protein (O'Farrell, 1975). Proteins were separated according to isoelectric point by isoelectric focusing in the first dimension gel, and according to molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis in the second dimension. Since these two parameters are unrelated, it is possible to obtain an almost uniform distribution of protein spots across a two-dimensional gel. In this study, the partially purified proteins (88 and 50 kDa) were further purified by two-dimensional gel electrophoresis separation. They were determined as single or multiple proteins by different isoelectric points (pIs). The 2-D system was conducted with Mini-Protean II 2-D cell of BIO-RAD laboratories. The procedure was done following the instruction manual. The mixture of partially purified proteins, 88 and 50 kDa (10 µl of each protein) was mixed with an equal volume of first dimension sample buffer and loaded directly on the first dimension gel surface. Alternatively, 5 µl of 2-D SDS-PAGE standard proteins was added to the sample mixture before loading. In this experiment the mixture of sample was loaded with and without 2-D SDS-PAGE standards comparatively. It's noted that the isoelectric points of the samples were interfered by the standard proteins, especially conalbumin and actin that had almost the same molecular masses. The samples and some standard proteins on second dimensional gel appeared to have streak bands along the gel in both conditions. The sample gels and the standard gel were matching on two-criteria. One is the absolute spot position and the other is relative spot positions and intensities. Furthermore, the migration distances of protein spots were measured comparing with standard proteins. Finally, the pI values of the 88 and 50 kDa protein antigens were approximately $\leq$ 4.5 to 5.6 and $\leq$ 4.5 to 5.1,

respectively. These results could not specify that the interesting proteins (88 and 50 kDa) were contaminated or not with other species of protein. Moreover, they could be recognized by IgG antibody in pooled sera of 10 AIDS patients infected with *P. marneffei* after separating on the two-dimensional gel and transferring onto nitrocellulose membrane. The immunogen profiles on nitrocellulose membrane were similar to their positions on the two-dimensional stained gel. In addition, the 25 µl of each crude proteins from *P. marneffei* 391H and 302BM were also separated on two-dimensional gel to compare with the 88 and 50 kDa spots position on the other gels. Their spot positions and relative intensities from these gels were similar. Immunoblotting of two crude protein antigens after separating on two-dimensional gel revealed that the 88 and 50 kDa proteins reacted with IgG antibodies and could be demonstrated along the gel from basic to acidic end, but their intensities were at the acidic end. The streak spots may be caused by antigens overloading or they had highly immunogenic property and also may have large carbohydrate content that can cause altered electrophoretic mobilities in SDS-PAGE giving anomalous results (Andrews,1965; Bretscher, 1971). In addition, SDS contamination in the sample may does adversely affect the linearity of the pH gradient (Sinclair; Rickwood, 1981)

D. Glycoprotein detection and lectin binding on nitrocellulose membrane

Polysaccharides are major components of the fungal cell wall. They have a role in structural functions to provide rigidity and physical stability to the fungus (glucan, chitin, cellulose) (Bartnicki-Garcia, 1968) and they also play a role in the regulation of virulence and dimorphism (Kwon-Chung and Rhodes, 1986; San-Blas, 1985). Polysaccharides and glycoproteins are also involved in antigenic recognition (Hearn, 1992; Hearn et al, 1990). Previous studies have demonstrated that glycoproteins purified from the water-soluble material of *A. fumigatus* mycelium are highly active in ELISA system and immuno-electrophoresis used for the detection of antigen/antibody binding (Le Pape and Deunff, 1987; Wilson et al, 1987). On SDS-PAGE combined with Western blot procedures showed that a wide range of molecules apparent molecular mass from approximately 20 to > 100 kDa had specific binding to antibodies raised in rabbits to *A.fumigatus* cell wall and cytoplasmic component. The ability to bind antibody was markedly reduced by treatment of these antigens with sodium periodate or with specific

proteases or glucanases. However, pretreatment *A. fumigatus* transbotted antigens with either concanavalin A (Con A) or wheat germ agglutinin (WGA), which bind extensively to these antigens, prior to allow reacted with anti-*Aspergillus* antisera could not inhibit antibody binding. This suggested that neither mannose, glucose or hexosamine residues are directly involved in the antigen/antibody binding (Hearn et al, 1990). However, much of the immunological reactivity appears to reside in the carbohydrate moiety, predominantly galactomannans of *A. fumigatus* (Dupont et al,1987; Van Cutsem et al, 1990), mannoproteins of *Cryptococcus neoformans* (Orendi et al, 1997), 65 kDa mannoprotein of *Candida albicans* (Gomez et al, 1996) and 3-O-methylmannose of *Coccidioides immitis* (Cole et al, 1991; Kruse et al, 1990)

In this study the partially purified 88 and 50 kDa protein antigens separated on two-dimensional gel were transblotted to nitrocellulose membrane following glycoprotein detection using Immun-blot kit (Bio-Rad laboratory). The result was observed on the intensity of dark blue purple color at protein spots position. The spots of 88 kDa protein gave stronger reaction than the 50 kDa protein. However, both of them were interpreted as positive for glycoprotein detection. This result might depend on the differences in carbohydrate composition of each molecule or the amount of loading proteins in the two-dimensional gel. To define antigens associated with glycoprotein nature, lectin binding was studied. The results showed that Con A bound strongly to 88 kDa protein and bound weakly to 50 kDa protein. This suggested that alpha-D-mannopyranoside or alpha-D-glucopyranoside residues were the major carbohydrate components of the 88 kDa antigen, whereas they were the minor components of the 50 kDa antigen. These carbohydrate residues may serve as antigen-antibody binding site of the both protein antigens. In addition, the crude culture filtrate concentrated proteins from three isolates of *P. marneffei* yeast form separated on one dimension SDS-PAGE and transblotted to nitrocellulose membrane were also studied with lectin binding. This result was similar to the other study of culture filtrate antigens of *A. fumigatus* by Makoto Kobayashi and Isao Miyoshi (1993). Con A showed strong binding to high molecular weight components (HMW) over 30 kDa but not binding to low molecular weight components (LMW) of approximately 18 kDa. Furthermore, WGA was also used in this study. The result showed that all of proteins (88 and 50 kDa) separated on the two-dimensional gel and transblotted to nitrocellulose membrane did not apparent bind with WGA. This suggested that these proteins did not have

N-acetylglucosamine or N-acetylneuraminic acid residues. However, WGA could bind with low molecular weight components of approximately 39-66 kDa of crude culture filtrate antigens from three isolates of *P. marneffei* yeast form after separating and electroblotting, respectively.

In conclusion, the present study demonstrated that the 88 and 50 kDa proteins were highly immunoreactive and secreted in large quantities into the culture filtrates of *P. marneffei* yeast form. The 88 and 50 kDa antigens have pI values of approximately $\leq$4.5 to 5.6 and $\leq$4.5 to 5.1, respectively, on the basis of two-dimensional gel electrophoresis technique. The isolation and identification of these two antigens by two-dimensional gel electrophoresis could not specify that they were single or multiple proteins because they had streaked on the second dimension gel (SDS-PAGE gel). However, these proteins could be identified to be glycoproteins by Con A binding property, a characteristic indicative of a mannoprotein or glucoprotein. These carbohydrate residues may serve as antigen/antibody binding site of both protein antigens. The importance of both carbohydrate and protein residues of these antigens in the antigen/antibody binding should be further studied. In addition, they should be further characterized for N-terminal sequences and immunological activity such as potent protective role or in the progression of the disease.