CHAPTER VI

DISCUSSION

Previous studies have been reported that mosquito released saliva proteins into human when its feeding for blood meal. These results have been proved by the detection of anti-mosquito saliva protein in human serum (116).

In this study, we hypothesized that people who have high risk of mosquito bite, will develop immune response by producing of anti-mosquito cell antibodies. We aimed to examine the anti-mosquito cell antibodies in human and experimental animal serum while many studies have indicated about anti-mosquito saliva proteins only. Three immunological assay were developed to detect antibodies in this experiment, including Western blot analysis, enzyme linked immunosorbent assay, and indirect immunofluorescence assay. In addition, RT-PCR assay was developed for the confirmatory method for dengue infection. In this study, the anti-mosquito cell antibodies in experimental animal are produced by immunization of New Zealand White rabbits with *Aedes albopictus* cell line, C6/36 cell. The serum were collected before and after immunization for detection of anti-mosquito cell antibodies. In addition, seventy human serum were collected from three source; 30 guards, 30 patients with dengue infection and 10 cord blood obtained from the King Chulalongkorn Memorial hospital. All serum were examined for anti-mosquito cell antibodies by the three developed assays.

In SDS-PAGE, different three source of mosquito cell proteins; whole body extracts of *Aedes aegypti* and *Aedes albopictus* mosquito and C6/36 cell extract, were used to react with rabbit's serum and human's serum. We have identified many proteins ranging between 10-175 kDa in these extract. The protein profile from three extracts are similar in pattern. The component with molecular weight of about 12, 14, 17, 26, 30, 35, and 41 kDa were dominant in all three extracts. We revealed that

different mosquito species expressed the same proteins as seen from separating of mosquito's whole body proteins by SDS-PAGE. The C6/36 cell line are cells from *Aedes albopictus* larva but showed reactive proteins as in adult. These proteins presumably represent structural proteins. However, the triplet of protein band from C6/36 cell extract, with molecular weight about 12-17 kDa, may be related with the developmental stage of *Aedes albopictus* mosquito because they are predominated in cell of mosquito's larva stage more than adult stage.

In animal study we try to simulate the natural mosquito bite by injecting antigen subcutaneously. Two rabbit were immunized with the continuous cell line of *Aedes albopictus* mosquito's larva stage, C6/36 cell. Post-immune rabbit's serum were examined for anti-mosquito cell antibody compared to the preimmune sera indicated that rabbits have been not exposed to mosquito bite before experiment. The rabbit were kept under mosquito net for the whole experiment duration. Thus the antibodies are induced by injection of C6/36 cells. Antibodies were observed after boosting. There were correlated between three assay, the markedly increasing of antibody were observed. Western blot analysis show multiple IgG-binding activity towards antigens of C6/36 cell while weakly reaction on protein extract of both *Aedes* mosquitoes. The different may be due to the different protein concentration from each sources of antigens. The protein with high antigenicity from C6/36 cell, have molecular weight of about 34, 47, 54, 65, and 83 kDa. By ELISA, it shows the sharp increase of anti-mosquito cell antibodies in post-immune sera. While the IFA show positive fluorescence on C6/36 cell. These results indicated that the rabbit's immune system was activated by C6/36 cell to produce the specific anti-mosquito cell antibodies. The mechanism of antibodies production against C6/36 cell is not known. We failed to detect injected mosquito cell from rabbit circulation by PCR method (data not shown).

For the detection of anti-mosquito cell antibodies in human serum, thirty guard's serum, thirty dengue patient's serum, and ten cord blood's serum were

included in this experiment. We observed that anti-mosquito cell antibodies from guards showed strong reactive in all assay types. The experiment in antibodies detection from serum of dengue patients with varies in age and clinical manifestation, showed weakly antibodies reaction to mosquito proteins. The frequently of positive results was different in the guards and dengue patients. Comparison between both groups, positive anti-mosquito cell antibodies were found in a significantly higher percentage of the 'Guards'. However, detection of anti-mosquito cell antibodies by Western blot analysis, the 32-35 kDa protein is the major protein on mosquito cell that have strong immunogenic epitope and antibody against this protein is present in almost of human serum that could detect anti-mosquito cell antibodies. This protein is a major component for antibody binding but the nature and significant of this protein is not known. The result of immunofluorescence assay in anti-mosquito cell antibodies detection corresponded with the result of Western blot analysis. When we compare the result of anti-mosquito cell antibodies from guard's serum and serum of patients with dengue infection, we found that the more intense bands and fluorescence are from guard serum more than serum of dengue patient. In ELISA study, in which the antibodies titer can be semi-quantitately measured, also showed that guard serum have significantly higher anti-mosquito cell antibodies than serum of dengue patient ($p<0.05$). By three assays, all results are consistent proved that guard have higher anti-mosquito cell antibody than dengue patient.

In addition, cord blood serum were tested for anti-mosquito cell antibody. They had not been exposed to mosquito before the time of blood sampling. Thus, they represented the best available negative samples. No antibodies reaction was observed in them (data not shown). Thus result also indicate that the passive transfer of anti-mosquito cell antibodies from mother to fetus is very low or can not be detected by our assay. However, the numbers of samples were low in group of cord blood due to the availability.

Results of this study suggested that mosquito cell from saliva can activated host immune response to produce the specific anti-mosquito cell antibodies. And we could detect these antibodies in human serum. The comparison between three group of human serum show that guard have most anti-mosquito cell antibodies while weakly antiboies reaction from dengue patient's serum and antibodies were absent in cord blood serum. In addition, by Western blot analysis, almost of human serum that could detect anti-mosquito cell antibodies presented antibody against 32-35 kDa protein. This protein may be the major protein on mosquito cell that have strongest immunogenic epitope. Moreover, from fluorescence assays the antibodies can recognized cytoplasmic protein, nuclear protein and cell membrane protein. It indicated that the immune response is varies in each person, some produced antibody specific to protein antigen in cytoplasm or nucleus or on cell membrane of mosquito cell. These proteins are not saliva protein as shown in previous studied (116). Moreover, in their studies the antigens they used were salivary gland instead of saliva. Thus, the antibodies could be antibodies to mosquito cells not the saliva proteins.

We compared the *Aedes aegypti* 's protein component between the whole body extract's protein from this study and mosquito saliva's protein that showed in the previous studied(116). Protein with molecular weight of 30 kDa was showed in both source. The 30 kDa protein presented in mosquito's saliva may be the mosquito's cell protein. Because of the previous study in snake showed the secretory epithelial cells from the lumina of the snake's venome gland might have been released during gland contraction(117). Thus, mosquito's cells were released from salivary gland into the saliva. Then mosquito's saliva was injected into the bitting-site during mosquito's feeding, so they activated to the human's immune system to produce the specific antibodies. Several studied showed proteins from mosquito's salivary gland or mosquito's saliva that recognized by antibodies from human serum with nearly molecular weight as shows in this study. The previous studied that showed mosquito

saliva's protein reacted with human IgE with molecular weight of 8, 18.5, 30.5, 33, 37, 50, 55.5, 61.5, 68 kDa. The studied about mosquito' salivary gland, *Aedes aegypti* 's salivary gland protein recognized by antibodies from one human serum with molecular weight about 14.9, 33.2, 40.3, 49.8, 61.7, 63.6, 67.6, 106.2 and 114.9 kDa (118). Another one previous studied, showed human IgG binding activity towards antigens with molecular weight of 12 to 23, 28 to 29, 31 to 34, 42, 62, and 78 kDa in extracts prepared from salivary gland (119). Due to, each studied used different standard protein and it's difficult to indicated the correctly molecular weight of proteins so these studied may be showed the same proteins recognized by human serum. So, most of proteins presented in mosquito saliva may be mosquito cell's protein and they were recognized by human serum.

Guards have a higher of anti-mosquito cell antibodies than patient with dengue infection, compatible with the view of a higher frequently of actual mosquito bites. The antibody level in guards might play same role in dengue infection. The neutralization activity from serum of rabbit and guard has been studied by plaque reduction neutralization assay. Unfortunately, the assay can not be done because the lack of adapted virus for cell culture.

In endemic area of dengue infection, the clinical manifestation may be asymptomatic or lead to a range of clinical presentation, even death. Population-based studies have shown increasing severity in the clinical features of dengue infection with decreasing age of the patient. Almost of patients with severe dengue infection are children with lower than 15 years old. Infant and young children may have a severe form of disease while the older children and adults may have asymptomatic infection or mild febrile syndrome. However, all age of traveler become infected with dengue while visiting endemic area. They always show the severity of clinical manifestation. So, it was likely that native adult have some immunity to protect them from the severity of disease. This protection may be anti-dengue antibodies from previous

infection or the anti-mosquito cell antibodies. The anti-mosquito cell antibodies may be one of factors to resist to dengue infection. As shown above, there are different level of anti-mosquito cell antibodies between person with high risk to mosquito bite and patient with dengue infection. The higher antibodies may be the principal factor to modified outcome infection. However, the different level of anti-dengue antibodies produced by each person might be the result of the variation of clinical severity.

The pathogenicity of dengue virus of dengue infection in endemic area are similar to the observation in Leishmaniasis. The native adult rarely to be infected with severe clinical manifestation while the infection in group of children or traveler show the severe form of disease. The studied in Leishmaniasis's vector, sand fly, by David Sack showed that while it obtaining blood meal, they salivated into the host's skin. And the study indicated that previous exposure to sand fly or immunity to salivary gland homogenate prevents Leishmaniasis infectivity. There was proposed that immunity to sand fly saliva may confer protection to subsequent Leishmania infection. As the observation in endemic area of Leishmaniasis, native adult have been exposed to uninfected sand fly bites for all of life, so they were activated to produced the anti-sand fly's saliva antibody, were effeciently protected them against the Leishmania. While children or the traveler no have immunity, so they were infected with Leishmania and showed the severe clinical sign. These studied indicated that prior exposure of mice to bites of uninfected sand flies conferred powerful protection against Leishmania major. The protections mechanism was associated with a strong delayed type hypersensitivity response. The study in experimental mice, they were vaccinated with constructed DNA vaccine containing the coding region of the main protein in salivary glands. After challenged with parasites plus salivary gland homogenate were efficiency protection. Both of pathology and the number of parasite were significantly reduced. This phenomenon as same in dengue infection.

Due to the mechanism of dengue replication by budding from host cells, mosquito cell, they must have some protein antigen of mosquito's cell on their envelope. The same as expression of major histocompatibility complex (MHC) class I gene products on human immunodeficiency virus (HIV)-infected cells and incorporated into the lipid enveloped of HIV virions (120). There are reported suggesting that anti-MHC immune responses can protect against HIV-1 infection comes from a macaque model in which immunization with a human lymphoblastoid cell line protected macaques against subsequent simian immunodeficiency virus (SIV) challenge, when the virus was grown in the same cell line (121, 122). Protection correlated best with antibodies against class I MHC (123). Studies by Arthur et al. (124) showed that class I and class II MHC is present on the envelope of HIV-1 and that antisera to these proteins precipitated intact virions. Chan et al. (125) also showed that immunization with purified class I HLA molecules can protect macaques from challenge with cell-free virus expressing the same class I HLA. Moreover, many studies have suggested that an immune response to human leukocyte antigen (HLA) alloantigens may contribute to protection against HIV infection. Fetal or newborn alloimmune responses directed at maternal HIV-1-infected cells or directed at free virus bearing maternal MHC determinants could also account for some children remaining uninfected (126). It is possible that HLA discordance provides protection of fetuses and infants from a variety of intracellular pathogens. If so, this would contribute to the maintenance of HLA diversity in populations. Therefore, the immunization against MHC/HLA alloantigens has been considered as a possible strategy for developing an AIDS vaccine. This suggestion follows from three observations: macaques model, structural homology was observed between HLA class I and classII and HIV-1 envelope proteins, and HIV-1 virions were shown to acquire and display more cellular proteins than envelope proteins. These findings support the potential to use an anti-HLA strategy in the development of an effective HIV-1

vaccine (127).

At the present, the pathogenesis of dengue infection is still controversial. Three theories, are frequently cited to explain the pathogenesis that occur in dengue infection with asymptomatic or mild to severe clinical manifestation. The most commonly accepted is known as the secondary infection or second infection with a heterogenous dengue virus serotype have a significantly higher risk for developing DHF and DSS. The second hypothesis assumes that there are some dengue virus serotype that have greater epidemic potential and severity of disease. The other hypothesis indicated that the nutritional status of patients with dengue infection related to the on going of severe pathogenesis. The study in Thailand by Usa Thisyakorn. et al, reported that evidence from every country in which DHF has been recorded indicates a strong association between good nutritional status and an increased risk of developing dengue shock syndrome. While dengue shock syndrome is rarely seen in children who live in the critically malnourished child.

Unfortunately, the hypothesis of each is not fully understood and lack of an animal model makes this a difficult area to study. The severity of disease in children may be explained in part by the social class and living condition. The malnourish children are living in poor condition and may have a high exposure to mosquito bite. The anti-mosquito antibodies in the children with different living condition should be studied compare to the antibodies in children living in better living condition and low exposure to mosquito bite.

Anti-mosquito cell antibodies may play a new role of the different clinical pathologic manifestation of the disease. The different anti-mosquito cell antibodies level may be related to the different pathogenesis of disease. However, the different level of anti-dengue antibodies produced by each person might be result of the variation of clinical severity.

Further research is necessary to investigate the different exposure to mosquito bites can have some impact to the pathogenesis of dengue infection by examination the antibodies level in group of infected children with mild syndrome compare to the group of children with severe clinical manifestation. In addition, the rabbit serum and human serum with strong reactivity on mosquito cell 's protein should to studied further about the neutralizing activity of anti-mosquito cell antibodies on infectivity of dengue virus by plaque reduction neutralization assay. It is also interesting to study the dengue infection people with hypersensitivity or allergic to mosquito bite. The present of specific antibody to saliva protein or mosquito protein may modified the outcome of dengue infection as it has been shown in the Leishmaniasis.