CHAPTER V

CONCLUSION AND DISCUSSION

HCV genotyping

The largest survey conducted to identify HCV genotypes in 1995 [12] found that throughout Europe types 1, 2 and 3 were the predominant genotypes infecting blood donors. In North America, type 1a was the most frequent genotype infecting blood donors in Pittsburgh; varying proportions of type 1a to 1b have been found in NANB patients elsewhere in the USA [149]. For the Far-East and Southeast Asia (SE Asia) major intra-region differences in genotype distribution are apparent in Japan and Taiwan, types 1b, 2a and 2b were the only variants detected , in Hong Kong a high frequency of type 6a infection was reported [150]. Type 3a was also found in Malaysia, Singapore and Thailand [10,12,19] and type 3 was also found to be the predominant genotype in Bangladesh, Pakistan and India. In South Africa, type 5a was the predominant genotype in blood donors. Now it has been established with reasonable certainty that type 4 is the predominant genotype in Zaire and elsewhere in Central and North Africa [9,15,150-151].

The variation in African and Southeast Asian regions appears to be distinct from that in Europe and other Western countries. Types 1a, 1b, 3a are the most frequent, the occurrence of additional genotypes in these countries is often attributable to recent travel or immigration; for example, a Canadian blood donor infected with type 6 was found to be a recent immigrant from Vietnam [152].

The purpose of the present study was to investigate HCV genotype distribution among Thai blood donors confirmed anti-HCV positive by third generation ELISA (Abbott Laboratories, Ill., USA) and HCV RNA positive by RT-PCR. There are numerous methods available to determine HCV genotypes such as RFLP, line probe assay (INNO LiPA) and direct sequencing to verify the genotypes of HCV based on nucleotide changes in different regions of Core, NS5, 5'NCR. As for the distribution of HCV genotypes in SE Asia, most data were obtained by conducting genotype assays such as

RFLP [10,12], INNO-LiPA [9], and assays using type specific primers [19,153]. By using these assays, only 50% of the genotype 6 could have been identified. In addition, it has been shown that INNO-LiPA HCV (Innogenetics, Ghent, Belgium) could not distinguish all virus subtypes or some novel genotypes discovered in Thailand and Vietnam, because the principle of this assay is typing based on the 5'NCR sequences of which were identical among genotype 1 and 6 [154].

The HCV NS5B region encodes an RNA-dependent RNA polymerase [155] which lacks proofreading activity. Consequently, many different but closely related variants are generated within the infected patient. The accumulation of such minor differences over time causes isolates to drift phylogenetically. Over prolonged periods these processes gave rise to multiple genotypes. The method used to determine the genotype and subtype distribution must be selected carefully because subtyping is required not only for the initiation and monitoring of therapy, but could also have implications for the choice of potential vaccine candidates [156].

In this study, two RFLP methods, direct sequencing in the core region and INNO-LiPA assay were performed for HCV genotyping. The INNO-LiPA method could not discriminate genotype 6a from genotype 1b (as shown in Table 7 and Figure 7). Since genotype 6a is commonly found in Southeast Asia and Thailand, INNO-LiPA assay was not suitable for genotyping HCV samples from this area.

From this study, direct sequencing seemed to be the most reliably method for HCV genotyping, especially for genotype 6a which cannot be distinguished from genotype 1b by using INNO-LiPA. For RFLP methods, in the past few years, our laboratory performed HCV genotyping by PCR-RFLP in the 5'NCR region [10] as previous described by Davidson et al [12]. This method needs seven restriction enzymes and the large amount of PCR products. Using this method, Davidson and his group could not detect genotype 6a from their samples which were isolated in Southeast Asia because of the almost identical in 5'NCR sequences of genotype 1 and 6a. That is genotype 6a samples were identified as genotype 1 by this condition. They performed another RFLP in a different part of HCV gene, core region, using samples that were previously identified as genotype 1. By using restriction enzymes *Ava* I and *Sma* I, genotype 6a could be distinguished from genotype 1.

In order to limit the amount of PCR product needed and to use fewer enzymes, the RFLP with slightly modification was performed in this study. RFLP was done in core region instead of 5'NCR and only three restriction enzymes, *Ava* I, *Sma* I and *Mbo* I, were used. Using *Ava* I and *Sma* I, Genotype 1 could be distinguished from others according to electropherotype patterns. To identify whether non-genotype 1 samples were genotype 3 or 6a, other restriction enzymes are needed. As previously described by Buoro et al [14], the use of 3 enzymes (*Acc* I, *Mbo* I, *Bst*N I) was sufficient to distinguish genotypes and subtypes. However, when only *Mbo* I was used in this study, HCV genotype 3 and 6 could be distinguished (as shown in Figure 8 and 9).

Although, certain genotypes can be identified using PCR-RFLP, the patterns obtained are quite difficult to interprete. In addition, only one base mutation at the restriction site can cause altered electropherotype pattern. Therefore, confirmation of RFLP by sequencing of core region is still needed for accurate typing.

Our data demonstrated the distribution of HCV genotypes among blood donors in Bangkok by using a reliable molecular method, direct sequencing, to characterize the HCV core region. These data did not represent the distribution of HCV genotypes found in Thailand because the selected cohorts were only come from Thai Red Cross, Bangkok. All blood samples were collected anonymously. No personal details ; sex, age, occupation etc was obtained, thus these data could not indicate the demographic pattern of HCV infection found in Thailand.

Difference from the previous study by Kanistanon et al in 1997 [19], they collected blood samples from blood donors in different parts of Thailand and performed genotyping by using type specific probes. They found that the HCV genotypic distribution from each study areas were the same. Even though using the different methods for genotyping, the distribution of HCV genotypes obtained from their study were the same as that of ours that were genotype 3, 1 and 6a, respectively. The HCV genotypic distribution was also the same as observed in the previous study by Theamboonlers et al [10] using PCR-RFLP method. These data provide the useful information for epidemiological studies, development of better diagnostic tools and new approach for treatment.

HCV specific immune response studies

In this study, we determined the HCV-specific response in HCV-infected blood donors. The liver function tests and liver pathogenesis were not examined in these subjects. Currently, only information of immune activity to HCV infection in chronic, acute and self-limited patients is available [99,157-159]. Therefore, it is quite interesting to investigate whether the immune activity in asymptomatic infected individuals is vigorous or multispecific.

Our study showed that nearly half of the HCV infected individuals exhibited responses to viral proteins. This information was established by assessing the HCV-specific PBMCs proliferative response and IFN-γ production *in vitro*, which presumably depends on the presence of T cells that have been exposed to antigen and undergone clonal expansion and cytokine production. However, these 2 read-out systems reflect different functions of the cells. Other studies showed that the proliferation was not correlated with antigen-specific cytokine production [160-161]. We used ELISPOT for detecting IFN-γ production instead of ELISA since this method is quite suitable for detecting cytokine production at a single cell level, so it could provide us additional information on the numbers of cells secreting that cytokine even at very low frequency [159].

The major disadvantage of the ELISPOT method is that scoring the wells for positive reactions involves the manual enumeration of large numbers of coloured spots, which can vary greatly in size and shape, thus, this method is subjected to operator bias [74].

A certain antigen can induce cell proliferation but not IFN-γ production and vice versa. In our study, one HCV protein induced cell proliferation but not IFN-γ production, which suggests that only cell proliferation assay may not be enough to specify whether an antigen could be a good candidate for HCV vaccine, especially when T_H1 response is required. In addition, a certain HCV protein can only induce IFN-γ production but not cell proliferation. This may not be a perfect candidate for a vaccine either, since cell proliferation is required for proper immune response.

According to our results, none of the infected blood donors showed both proliferation and IFN-γ production induced by NS5 and none of donors showed only response to NS5. This indicated that NS5 may not be an immunodominant epitope compared with core and NS3/4. Some samples gave no response with all HCV proteins used in this study by both methods. However, it could not be concluded that there was no HCV specific immune response in these donors because HCV proteins used in this study were not all proteins encoded by the entire HCV genome. The portions that were not included in our study were envelope proteins (E1, E2) and also NS2 protein. Because of hypervariable properties of an envelope protein, it was very difficult to generate SOD-E1E2 for using as antigen.

Interestingly, more than 80% of blood donors infected with HCV genotype 1a did not response to all proteins used even though the antigens were derived from HCV 1a genotype. This may be due to the defect of their immune system in response to HCV antigen. Since there was no information on liver manifestation of donors, we still could not conclude whether the unresponsiveness was due to the advance stages of liver pathogenesis.

According to our data, NS3/4 seemed to be the most immunogenic portions among proteins used in this study. It is not surprising to us because other studies (shown in Table 2 and 3) also reported similar results. NS3 protein is one of the most conserved protein among subtypes of HCV. A very early T cell proliferative responses to HCV NS3 appeared to be associated with viral clearance and antibody titers increased by over 1,000 fold as well because NS3 contains both T and B cell epitopes. NS3 functions as protease, helicase and NTPase. These vital enzymatic functions may explain the limited sequence variation within the NS3 region and suggest that the NS3 protein may constitute a good therapeutic vaccine candidate [162].

Hepatitis B virus is another virus causing liver diseases and it is known to be exquisitely sensitive to the antiviral effects of IFN-γ [163-167]. For HCV, according to previous reports, HCV persistence is not due to a failure of the T_H1 cells to produce cytokines but the persistence of virus may be due to the viral resistance antiviral effects of IFN-γ. In addition, the low of numbers of HCV-specific $CD4^+$ and $CD8^+$ T cells in the

peripheral blood and in the liver may be another fundamental explanation for virus persistence during HCV infection.

HCV-specific $CD4^+$ T cells are multispecific, compartmentalize to the liver, and produce IFN-γ [159]. When percentage of HCV specific responses studied by several groups were compared, we found that 36% of asymptomatic infected blood donors in our study showed positive response; whereas 11% of chronic subjects in Schirren study, 16% of chronic and 64% of recovery subjects in Chang study showed HCV specific response [159,167]. These data suggest that a low frequency of virus specific T cells is associated with failure to clear the virus and chronic course of disease. We are the first to demonstrate the response in asymptomatic infected Thai blood donors and the percentage of subjects giving positive response from our study was between chronic and recovering subjects.

Recently, Sugimoto et al [168] performed the same methods (proliferation and IFN-γ ELISPOT) to study HCV specific responses and showed that the race or ethnic origin of the patients contributed to the clinical outcome and also cellular immune response. They examined the immunologic basis for the apparent ethnic difference in clinical outcome of hepatitis C virus infection between African Americans (AA) and Caucasian Americans (CA). They found that AA ethnicity was associated with a significantly greater CD4-proliferative T cell response to HCV, particularly to the nonstructural antigens (22% AA vs 0% CA) as well as better clinical parameters of liver disease. They also found that most HCV-specific $CD4^+$ T cell proliferative responses in AA patients were uncompanied by concurrent IFN-γ production, suggesting a dysregulated virus-specific, $CD4^+$ T cell effector function during chronic HCV infection. In our study, we found the similar result. In most of samples that gave positive results by proliferation assay, IFN-γ production assay was negative. However, we were not able to conclude that IFN-γ detected in some samples were from $CD4^+$ T cells as concluded by Sugimoto group since CD4 depletion was not performed in out study.

In our opinion, antigens that are able to induce cell proliferation and IFN-γ production are promising for further study. Since different blood donors showed different response to our HCV proteins used, it is quite interesting to investigate whether a combination of antigens can give a better response. In addition, 51.2% of infected

blood donors showed no response to all proteins used, so valuable information could be obtained if all individuals in this group are followed with respect to their health status. Additional candidate HCV proteins should be tested to see whether an increased number of subjects showing a positive response, could be obtained.

There was no significant difference between different genotypes in response to HCV antigens used. Higher numbers of subjects infected by genotype 6 are needed to investigate the correlation between genotypes of virus and host immune response, in more detail.

For HCV infection, a low frequency of virus-specific T cells is associated with failure to clear the virus and chronic course of disease [169]. CTL studies have been difficult to perform because of the relatively low frequency of CTL precursors in the peripheral blood. HCV specific responses are compartmentalized to the liver so in this study we observed HCV specificity by generating T cell lines from intrahepatic T cells. Because of low frequency of specific T cells, as read-out system for HCV-specificity, the $CD8^+$ T cell numbers are determined either by measuring secreted IFN-γ with the enzyme-linked immunospot (ELISPOT) assay or by cytoplasmic staining for IFN-γ and subsequent fluorescence-activated cell scanning analysis. This new method allows detection of cytokines in an antigen-specific manner at a single cell level and is therefore very useful in determination of virus-specific T lymphocytes at very low frequency [169].

Development of antigen expressing system

One of the objectives of HECSA project (Hepatitis C vaccine efficacy against South East Asian genotypes) is to study the cross-reactivity of T cell between individual infected with different HCV genotypes. Autologous BLCL infected with recombinant vaccinia viruses encoding parts of the HCV genome (rVV-HCV) are generally used as a tools for studying of CTL activity. The disadvantages of using such rVV-HCV include VV infectivity, cytopathic effect and difficulties in generating the recombinant viruses. To decrease the infectivity of the virus, BLCLs were fixed with some substances before incubating with T cells. The fixation process is time-consuming and inconvenient. The process to generate rVV is also difficult. Recombinant DNA recombination which leads

to the insertion of foreign DNA into VV genome occurs at a very low frequency; only about 0.1% of the progeny carried foreign genes [170]. Cloning the gene into the plasmid was more convenient and practical which allows expression of different HCV genes of different genotypes.

In the present study, we have shown that the EBV vectors are actually extremely effective in transfecting genes into BLCLs when compared with CMV-based promotor plasmids. The EBV-based plasmids used in the present study, pNS vector, carried the oriP region from EBV as *cis* elements for DNA replication. After transfection into BLCLs, EBNA-1 from EBV in BLCLs bind to oriP which facilitates the retention and replication of the plasmid DNA, making pNS vector a very efficient expression vector [136].

VR1012Neo$^+$ vector, another eukaryotic expression plasmid utilizing CMV based promotor was not suitable for BLCL transfection even though it has a high expression efficiency in other cell lines such as NKNT-3 cells (as shown in Figure 18). The expression of NS3 protein in NKNT-3 cells by VR1012Neo$^+$ was higher than by pNS/N16 vector but it was vise versa in BLCLs. We repeated the transfection experiment 3 times but nearly all BLCLs died 3 days after transfection with VR1012Neo$^+$ (before a selection protocol). It is uncertain what caused this result. It was likely that the transfected BLCLs could not stand high expression of foreign proteins in the cells. We concluded that pNS vector is better than VR1012Neo$^+$ for BLCL transfection.

When comparing rVV-HCV with pNS/N16, the level of NS3 expression in BLCLs infected with rVV-HCV was higher than that of transfected BLCLs (12% versus 6%). However, the anti-HCV cellular activities induced by transfected BLCLs was similar to that of BLCLs infected with rVV-NNRd. The number of IFN-γ spots generated from T cells incubated with both types of stimulator cells and also % of specific T cells as measured by FACS analysis were indifferent.

There are many advantages of using the pNS vector. First, it is suitable to transfect BLCL. Second, it is easy to insert different foreign genes into the plasmid. Third, it is easier to handle than rVV.

Unfortunately, we had only 1 T cell line to test with this plasmid. Thus, more work is needed to confirm whether plasmid transfection can be used to replace the conventional technique for detection of CTL.

When we used pNS/N16 to test the anti-HCV activity in PBMCs from 3 blood donors previously shown positive with NS3/4 protein by proliferation and IFN-γ production tests, we could not detect positive IFN-γ spot in any samples. The reason could be that there were no specific T cells or very low frequencies of specific T cells in the PBMCs. From the previous studies [171-172] it was apparent that PBMCs must be *in vitro* activated with specific antigen before detection because of the very low frequency of specific T cells. In addition, having a proliferative response and IFN-γ production by PBMCs for NS3/4 protein may not necessarily implicate the presence of a specific CTL against the same antigen. The other reason of negative result may be due to very low NS3 expression in BLCLs. This reason is not likely because NS3 expression in transfected 816 BLCLs, being only 6%, was able to stimulate the HCV specific T cells in the previous experiment.

The reason for having high background of IFN-γ spots in the control well (non-transfected BLCLs incubated with PHA-stimulated PBMCs) may come from 2 possibilities. First, polyclonal activation by PHA as used in this study may allow the proliferation of all T cell clones so EBV-specific T cells were increased. Normally, there was quite low frequency of HCV specific T cells in PBMCs, therefore the proliferation of other T cell clones might mask the activities of HCV specific T cell clones. The other possibility was from the natural HCV protein expression in BLCLs. It has been previously shown that HCV has the extrahepatic site of replication in B cells and some peripheral cells [173]. In addition, EBNA-1 protein from EBV supported HCV replication [174]. In this study, BLCLs were generated from EBV infected B cells, thus EBNA-1 was present in BLCLs. If these B cells had HCV infection, EBNA-1 would help facilitating HCV replication that could be resulted in the present of HCV protein on the cellular surface.

The effect of EBV seemed to be less when the samples used were LILs. For detection of HCV specific T cell activities, it is quite easier to detect HCV specific T cells in liver than in PBMCs because the major population of LILs were antigen specific T cells. The HCV specific T cells in the liver were 30 folds more than that of in PBMCs so the activity of specific T cells found in liver can overcome all background spots from non-specific T cells observed when test in PBMCs. This will be the limitation of using BLCLs as stimulator cells to detect HCV specific T cell activities in PBMCs.

In conclusion, data from this study provide the information on HCV distribution among Thai blood donors collected from Bangkok, the HCV specific response of PBMCs from asymptomatic infected individuals and finally, the preliminary data suggest that the plasmid carrying a gene for an HCV protein may be used as an alternative for recombinant vaccinia virus in studying of anti-HCV cellular activity. All information obtained will be useful for epidemiological study, vaccine development and study of immune response against HCV.