CHAPTER I

INTRODUCTION

BACKGROUND AND RATIONALE

Hepatitis C virus (HCV) is an important human pathogen that chronically infects more than 3 % of the world's population [1]. HCV has infected an estimated 170 million people, which is more than five times higher than HIV [2]. HCV causes a wide spectrum of liver diseases ranging from asymptomatic or symptomatic acute infection with self-limited disease to persistent infection with chronic active hepatitis and an increased risk of liver cirrhosis and hepatocellular carcinoma [3]. Twenty percent of people infected with HCV will overcome the virus during the initial infection and clear it from the bloodstream. The remaining individuals will develop a chronic infection; 10% to 20% of these develop cirrhosis and 1% to 5% develop a liver cancer called hepatocellular carcinoma [2].

HCV has been classified as a member of the *Flaviviridae* family. The other members of this family include the classical flaviviruses (such as yellow fever and dengue viruses) and pestiviruses (such as bovine viral diarrhea virus). All members of this family are small-sized, enveloped viruses containing a positive-sense single-stranded RNA encoding a viral polyprotein. The viral genome of HCV is approximately 9.6 kilobases long and contains a single open reading frame (ORF). The ORF is flanked by 5' and 3' untranslated regions (UTRs). The HCV polyprotein is cleaved by cellular and viral proteinases into 10 different products, with the structural proteins located in the amino-terminal third and the nonstructural replicative proteins in the remainder [5]. The similarity between HCV and flavivirus is that their genomes consist of a single polyprotein which is cleaved into structural proteins (capsid: C, envelope glycoproteins :E1E2) and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B). HCV and pestiviruses share a similarly structured of internal ribosomal entry site in the 5' noncoding region (5' NCR). Amino acid sequence similarity between HCV, pestiviruses

and flaviviruses has been detected in the region surrounding the active site of the RNA polymerase [4].

HCV shows a high degree of genomic variation during the course of infection, resulting in the distribution of a number of variants or quasispecies [6]. Scientists have identified more than 100 strains of the virus and grouped them into six major genotypes which tend to cluster in different regions of the world. HCV 'isolates' with a nucleotide homology lower than 66-69% and 77-80% are considered to belong to different genotypes and subtypes, respectively. Strains having 90% or greater homology are considered variants within the same subtype [7]. Several studies have demonstrated that none of the six HCV genotypes appears to be more pathogenic than the others [2,6].

Current methods for routine subtyping employ different approaches for analyzing PCR fragments. Methodologies include type-specific amplification of the core or NS5B regions[8], or analysis of 5'NCR, core or NS5B fragments by hybridization to type-specific probes [line probe assays (LiPA)] [9], restriction fragment length polymorphism (RFLP) [10-14], direct sequencing and grouping by phylogenetic analysis [15].

The final outcome of infections by viruses that cause chronic diseases is believed to depend mostly upon the balance between the rate of replication of the infecting virus and the capacity of the immune system to mount rapid, multispecific and efficient virus-specific responses to inhibit infection before the virus can devise strategies to evade immune surveillance. For viruses with a high mutation rate such as HCV, all factors which delay the onset of antiviral activity can allow the virus to escape neutralizing responses or to interfere with the host's protective mechanisms through its continuous genomic variation. Therefore, the inability of the immune system to rapidly neutralize infection gives the virus time to develop strategies to escape protective responses and to create conditions that can result in persistence even in the face of an active antiviral response [16]. Due to the serious problem caused by HCV, there is a pressing need to develop vaccination strategies aimed at preventing and possibly eradicating HCV infection.

Several major practical and scientific problems arise in designing an HCV vaccine. First, HCV is only readily detected as RNA by RT-PCR. Second, the only species that can be infected by HCV are humans and chimpanzees. Third, the virus

does not replicate efficiently *in vitro.* Fourth, some viral proteins have very high mutability. Further more, there is little information on correlation between immunity and HCV pathogenesis [17].

An HCV vaccine should be capable of either preventing infection or preventing the development of chronic infection following acute infection. Another essential requirement for an HCV vaccine is that it must protect against the major circulating genotypes. An ideal HCV vaccine should prime cross-neutralizing anti-envelope antibodies, and provide broad HCV-specific helper and inflammatory $CD4^+$ T cell responses, as well as HCV-specific cytotoxic $CD8^+$ T cell responses. The $CD4^+$ T cell response to viral proteins is usually essential for protection, since these cells are needed to help antibody production by B cells, and support priming of $CD8^+$ T cells specific for virus-infected cells. T lymphocyte responses to viruses may have two opposing effects. They may be crucial for protection, or can be harmful, mainly through $CD8^+$ T cells, which damage infected tissues in an attempt to clear the virus.

At present, vaccine trials are based on the HCV 1a sequence [18] because it is the genotype that is commonly found in the United States which is the first isolated genotype. The predominant HCV genotypes found in Thailand ranked from the most prevalent are 3a, 1b and 6a, respectively [10,19]. However, there is no information on T cell activities in HCV infected subjects from Thailand against HCV 1a proteins. In addition, the study of $CD8^+$ T cells is also a problem due to the low frequency of these cells in peripheral blood [20]. Therefore, we need an efficient way to present HCV protein to T cells and a highly sensitive method to detect HCV-specific T cell response [21-22].

In this study, we analyzed activity of T cells from HCV-infected blood donors against HCV1a proteins. The benefit of this study will be the ability to predict the immunodominant regions in HCV proteins for use in further vaccine development.

Hypothesis

The essential requirement for an HCV vaccine is that it should elicit broad immune response against the major circulating genotypes. At the present time, most of the studies are based on the proteins from HCV1a genotype. According to the

conservation of immunodominant parts, PBMCs from blood donors infected with different HCV genotypes found in Thailand showed cross reactivity against HCV1a proteins.

Objectives

1. To compare RFLP, INNO-LiPA and sequencing methods for genotyping HCV virus
2. To characterize HCV genotypes presented in HCV-infected blood donors
3. To study immune responses against HCV 1a proteins of PBMCs from blood donors infected with different HCV genotypes

Conceptual Framework

I. Comparison of RFLP, INNO-LiPA and sequencing methods for genotyping HCV virus

17 chronic HCV patients, 18 blood donor samples

RT-PCR to amplify HCV core region

PCR product

Characterization of HCV by

- 2 RFLP methods
- INNO-LiPA
- Direct sequencing

Results are compared and suitable method is selected

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II Characterization of HCV genotypes presented in HCV-infected blood donors

Blood donor samples positive for anti-HCV and RT-PCR for HCV RNA

Genotyping of samples by a method selected from I

III Study of immune responses against HCV1a proteins of PBMCs from blood donors infected with different HCV genotypes

1. Study immune response using soluble HCV 1a proteins (core, NS3/4 and NS5)

PBMCs from blood donors infected with HCV
(genotypes were already characterized in II)

Proliferation and IFN-γ production assays against core, NS3/4 and NS5

2. Study immune response using BLCL transfected with plasmid expressing HCV NS3/4

2.1 Construction of plasmid expressing HCV NS3/4 protein

2.2 Transfection of BLCL with plasmid expressing NS3/4 protein

2.3 Immune response against BLCL plasmid expressing HCV NS3/4 protein

Liver infiltrating lymphocytes or PBMC from HCV infected individuals

Stimulation with BLCL plasmid expressing HCV NS3/4 protein

IFN-γ production detection

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Assumption

All blood donors including in this study were collected anonymously from The National Blood Center of Thai Red Cross, Thailand. Historical and liver manifestation data cannot be defined.

In the collecting process of whole blood, the anticoagulant used is Citric Phosphate Dextrose (CPD) which is different from general anticoagulant used for PBMC study (EDTA/heparin). The effect of different anticoagulants used on the activity of PBMCs was not determined.

Key Words

Hepatitis C virus (HCV), Proliferation, IFN-γ production, Genotype, EBV-based plasmid

Operational Definition

For the proliferation test; SI $\geq$ 3 and Δ cpm > 2000 were considered to be positive.

A significant response to HCV determined by ELISPOT to detect IFN-γ production was defined if the number of positive spots in the well that contained HCV antigen was 2 times more than the number of spots obtained in the presence of the control antigen.

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Limitation

All PBMCs used in this study are frozen specimens so the activity of the cells such as lymphocyte proliferation, IFN-γ production are less than the activity of the fresh ones. The other point is that the number of the PBMCs are quite limited. We cannot collect the new batch of blood from the same donor at different time course because the

immunological status is different. For immunological test operated in Thailand, it is very difficult to perform because there is no HCV antigens (both recombinant proteins and vaccinia encode HCV gene) used for testing.

Expected Benefits

1. Obtaining information on the prevalence of HCV genotypes in blood donors.
2. Mastering the regions of HCV 1a proteins which induce HCV specific response (immunodominant) in blood donors infected with different HCV genotypes. The benefit of this study will be used for further vaccine development.
3. Obtaining information on cross-reactivity against HCV 1a proteins.
4. Expressing HCV proteins by using EBV-based plasmid instead of recombinant vaccinia HCV construct for testing T cell activity.

Research Methodology

1. Sample collection

   Whole blood samples: 100 blood donors collected from The National Blood Center, Thai Red Cross, Thailand.

   Chronic HCV patients : 10 from Rotterdam, The Netherlands, 7 from Pha Monkutklao hospital, Thailand.

   Healthy donors: 10 anti HCV negative blood donors, collected from Blood Bank in Rotterdam, The Netherlands, without a clinical history of hepatitis and symptoms or signs of liver disease.
2. Process of study
   - whole blood collection
   - isolation and plasma collection
   - molecular characterization of HCV RNA
   - proliferation and IFN-γ production test
   - generation of T cell lines and BLCLs
   - cloning, transfection and expression of HCV NS3 protein

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- HCV specific T cell detection

3. Data collection and analysis

Descriptive data analysis was used to calculate the percentage of HCV genotype prevalence. The differences between the prevalence of T cell responses to HCV proteins in each genotype were analyzed using a contingency table (Chi-Square test) or Fisher's exact test with two degrees of confidence. Values of $p < 0.05$ were considered to be statistically significant.

Phylogenetic analysis was performed by the PHYLIP package, version 3.57c (J.Felsenstein, Department of Genetics, University of Washington)

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