

Modelling of a self-adjuvanting lipopeptide vaccine SISOR-P3 for immunization against HCV

Team Number : 5

Introduction

Hepatitis C is a blood-borne, infectious viral disease caused by the hepatotropic Hepatitis C virus (HCV). Acute hepatitis is often asymptomatic, and though it can be cleared, is often left untreated. Chronic hepatitis leads to cirrhosis of the liver and is the leading cause of liver transplantation in the United States. Hepatitis C was discovered in the mid 1970's when post-transfusion patients were being diagnosed with hepatitis cases not caused by either hepatitis A or B. This new virus was given the name non-A, non-B hepatitis (NANBH), and for nearly a decade any efforts to identify the exact virus failed. It wasn't until 1989 that NANBH was renamed hepatitis C (Wikipedia, 2007).

Pathogen Summary

Hepatitis C virus is a small, enveloped single-stranded, positive sense RNA virus in the *Flaviviridae* family. The genome is approximately 10,000 nucleotides and encodes a single polyprotein of about 3,000 amino acids. This polyprotein is processed by the host cell and viral proteases into three major structural proteins and several non-structural proteins necessary for viral replication. Several different genotypes of HCV with slightly different genomic sequences have been identified that correlate with differences in response to treatment with interferon-alpha (Worman, 2002).

About 85% of individuals acutely infected with HCV become chronically infected. Hence, HCV is a major cause of chronic hepatitis. Once chronically infected, the virus is almost never cleared without treatment. In rare cases, HCV infection causes clinically acute disease and even liver failure, however, most instances of acute infection are clinically undetectable (Worman, 2002).

The natural history of chronic HCV infection can vary dramatically between individuals. Some will have clinically insignificant or minimal liver disease and never develop complications. Others will have clinically apparent chronic hepatitis. Of these, some go on to develop cirrhosis. About 20% of individuals with Hepatitis C who do develop cirrhosis will develop end-stage liver disease. Cirrhosis caused by Hepatitis C is presently the leading indication for orthotopic liver transplantation in the United States (Worman, 2002).

A major problem in discussing prognosis in patients with chronic hepatitis is that it is difficult to predict who will have a relatively benign course and who will go on to develop cirrhosis or cancer. Certain findings on liver biopsy can be helpful in predicting a relatively benign or progressive course. Viral genotype may also play a role (Worman, 2002).

Pathogen Structure:

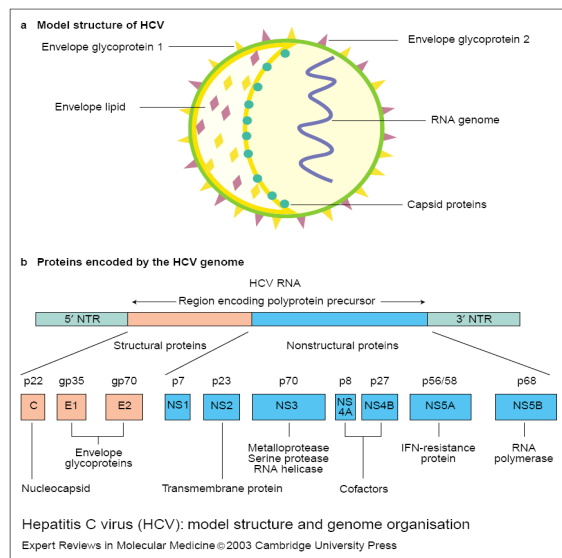


Figure 1. Hepatitis C virus (HCV): model structure and genome organisation. (a) Model structure of HCV. The left-hand side of the illustration shows the viral surface of envelope lipids and glycoproteins; the right-hand side shows the RNA genome encased by capsid proteins. (b) Proteins encoded by the HCV genome. HCV is formed by an enveloped particle harbouring a plus-strand RNA of ~9.5 kb. The genome carries a long open-reading frame (ORF) encoding a polyprotein precursor of 3010 amino acids. Translation of the HCV ORF is directed via a ~340 nucleotide long 5' nontranslated region (NTR) functioning as an internal ribosome entry site; it permits the direct binding of ribosomes in close proximity to the start codon of the ORF. The HCV polyprotein is cleaved co- and post-translationally by cellular and viral proteases into ten different products, with the structural proteins [core (C), E1 and E2] located in the N-terminal third and the nonstructural (NS2–5) replicative proteins in the remainder. Putative functions of the cleavage products are shown.

(Figure reprinted from Anzola and Burgos 2003)

Disease Mechanism

Hepatitis C virus (HCV) is the major causative agent of chronic non-A, non-B hepatitis. The life cycle of HCV is largely unknown because a reliable culture system has not yet been established. HCV presumably binds to specific receptor(s) and enters cells through endocytosis, as do other members of the Flaviviridae family of viruses. The viral genome is translated into a precursor polyprotein after uncoating, and viral RNA is synthesized by a virus-encoded polymerase complex. Progeny viral particles are released into the luminal side of the endoplasmic reticulum and secreted from the cell after passage through the Golgi apparatus (Moriishi and Matsuura, 2003).

Acute hepatitis caused by HCV is usually subclinical. It has been reported that spotty necrosis/degeneration of hepatocytes and mixed inflammatory infiltrates in lobular parenchyma and portal tracts can be observed in acute hepatitis. Dense lymphoid aggregates in the portal tracts, inflammatory bile duct damage, and microvesicular steatosis were also found. A strong CD4⁺ proliferative and cytokine response to the HCV nonstructural protein 3 was correlated with viral clearance in acute HCV infection. In the chimpanzee model, animals that progressed to chronic HCV infection showed no detectable or had a narrowly focused HCV-specific CD8⁺ cytotoxic T lymphocyte (CTL) response, whereas animals that cleared HCV infection had early multispecific CTL responses that were durable for at least one and a half years. The data implies that the

host immune response may play a role in determining the clinical outcome in acute hepatitis (Lau, 1998).

Chronic hepatitis causes insidious and progressive liver damage in most patients. Chronic viral hepatitis causes a diffuse necroinflammatory reaction, lymphoid aggregates in portal tracts, epithelial damage of small bile ducts, and microvesicular and macrovesicular steatosis of hepatocytes. A small but increased number of hepatocytes show features of apoptotic liver cell death. Liver fibrogenesis is enhanced which contributes to the fibrosis and cirrhosis seen in late stages of chronic hepatitis (Lau, 1998).

A B cell response [or antibodies to HCV (anti-HCV)] has been detected in most patients with chronic HCV infection. The fact that most patients develop chronic infection in the presence of anti-HCV suggests that such antibodies fail to induce viral clearance. In the chimpanzee model, neutralizing antibodies were shown to be highly strain specific (and even quasispecies specific) and not protective against heterologous or even autologous challenge. From the perspective of disease pathogenesis, anti-HCV may cause liver damage by recognizing HCV antigen (or autoantigen) on the surface of infected cells or through immune complex deposition. HCV antigens have been detected in the cytoplasm of infected cells but not on cell membranes both *in vivo* in patients' livers as determined by immunohistochemistry and *in vitro* using a high-level recombinant vaccinia expression system. Immunoglobulin deposition was found to be uncommon in the liver of patients with chronic hepatitis C (Lau, 1998).

Pathomorphological studies have shown occasional acidophilic bodies and hepatocyte dropout, features that are compatible with apoptosis in patients with chronic hepatitis C. This was confirmed by *in situ* terminal transferase labeling. Host immune CTL-mediated pathways of apoptosis are activated, suggesting that apoptosis of liver cells may at least partly be related to the host immune defense. The presence of a normal HCV carrier with normal liver histology and the observation that isolated hepatocytes with cytopathic changes are uncommon suggest that direct viral cytopathicity does not contribute significantly to liver damage in chronic hepatitis C (Lau, 1998).

Immune Response

The non-specific mechanisms of the innate immune response are the first line of defense against HCV infection. This response consists of interferon production and NK cell activation. A study in chimpanzees showed NK and NKT cells are capable of eliminating an HCV infection without a HCV-specific T cell response (Thomson *et al*, 2003). Also, IFN α exerts potent and rapid antiviral effects on HCV (Gremion and Cerny, 2005). Finally, Kupffer cells have been shown to have antiviral activity by producing cytokines and nitric oxide (Cavanaugh *et al*, 1997). Thus in an acute infection, the innate immune response is critical for viral control. However, it is often asymptomatic, and infection is usually not detected until several weeks after exposure. In fact, one way HCV evades the immune response is to inhibit the activation of IFN α (Li *et al.*, 2005)

HCV is able to elicit both a humoral and cellular immune response. After an incubation period of 3-12 weeks, symptoms become recognizable, and within four weeks, antibodies against both structural and non-structural HCV proteins can be detected (Gremion and Cerny, 2005). Neutralizing antibodies are produced against the hypervariable region 1 (HVR1) of the E2 envelope protein (Farci *et al*, 1996). However, HVR1-specific antibodies are highly strain specific; thus they are not effective against more than one genotype (Gremion and Cerny, 2005). Another downfall of the humoral immune response is that HCV antibodies are incapable of inducing long lasting protection. HCV-specific antibodies are undetectable 18-20 years after recovery from a previous infection (Gremion and Cerny, 2005). Even though a humoral response is induced, it is of relatively low titer and restricted to IgG1 subclass antibodies (Netski *et al*, 2005). But recently reports suggest the existence of neutralization epitopes which are more conserved and have broader neutralization capacity (Bartosch *et al*, 2003).

In the cellular immune response, both CD4⁺ and CD8⁺ T cells play an important role in HCV infection. Studies have shown that a strong and multi-specific CD4⁺ and CD8⁺ T cell response is required for the elimination of HCV in acutely infected patients. CD4⁺ T cells regulate antigen-specific B cell activity (a Th2 profile) as well as secrete lymphokines to regulate CD8⁺ T cell responses (a Th1 profile), which then leads to the production of Th1 cytokines (IL-2, IFN- γ and TNF α) (Gremion and Cerny, 2005). Viral clearance is more likely to occur when HCV patient display a Th1 profile upon antigen-associated stimulation rather than a Th2 profile and Th1-type CD4⁺ T cells are maintained long after elimination of the virus (Tsai *et al.*, 1997, Sugimoto *et al.*, 2003). Therefore, a Th1 profile may generate a more protective immune response. This memory response has been shown to provide protection against re-infection (Gremion and Cerny, 2005).

CD8⁺ T cells are necessary for the direct killing of infected cells and for secreting the antiviral cytokines IFN- γ and TNF α . During the first six months of infection, a multi-specific CTL response is sufficient to control the virus; however, it is unable to clear it (Gremion and Cerny, 2005). In order to sufficiently eliminate the virus, Th1 immunity combined with enhanced CD8⁺ T cell activity is required. However, the enhanced CD8⁺ response occurs with a long-lasting inflammatory secretion, which is responsible for the development of cirrhosis and hepatocellular carcinoma (Gremion *et al.*, 2005).

Epidemiology

HCV affects over 170 million individuals worldwide and is the most common blood-borne infection in the United States (Rustgi, 2007). In a 1994 survey of the United States, approximately 3.9 million individuals who were infected with HCV were shown to have antibody to HCV, and approximately 2.9 million individuals who tested positive for HCV RNA were chronically infected (Rustgi, 2007). When the United States was again surveyed in 2002, the number of individuals showing HCV-specific antibody had risen to 4.1 million, and the number of chronically infected individuals who tested positive for HCV RNA had risen to 3.2 million (Rustgi, 2007). Presently, it is estimated that approximately 30,000 new cases of HCV infection occur every year and 85% of those infected with

HCV will develop a long-term chronic infection. Finally, nearly 20% of chronically infected individuals will develop cirrhosis of the liver in approximately twenty years. In fact, in one year 8,000 to 10,000 chronic liver disease deaths are due to HCV in the U.S. alone (Rustgi, 2007).

The main risk factors for infection are associated with blood-to-blood contact. Therefore, there are certain subpopulations at higher risk than the general public. This includes military veterans, healthcare workers; incarcerated individuals or other institutionalized individuals, the homeless, or members of households with HCV-infected individuals. Also, children born to HCV-positive mothers may be infected *in utero* or during birth. Individuals receiving blood transfusions or organ transplants before 1992, injection drug users, and those involved in high-risk sexual behavior are also at a risk for contracting HCV (Rustgi, 2007).

Sequence analysis of the E1 envelope gene in HCV isolates from around the world has discovered that at least six major genotypes exist, and each major genotype is divided into several subgroups. Infection by genotype 1 is more prevalent in the northeastern, southeastern, and mid-western sections of the United States. Also, African Americans are more likely to contract a genotype 1 infection than Caucasians, Hispanics, or Asian Pacific Islanders. Genotypes 2 and 3 are also highly prevalent in the Americas and Europe. Around the world, genotype 4 appears commonly in the Middle East and Africa; genotype 5 occurs most frequently in South Africa, and genotype 6 occurs most frequently in Southeast Asia. However, recent surveys have shown genotype 4 to be rapidly spreading in Western countries, mainly among injection drug users (Rustgi, 2007).

Treatment

There is currently no human approved HCV vaccine. The high variability and high mutation rate has made the development of an effective vaccine difficult. Currently, the only treatment option is therapeutic and consists of a combination of interferon and ribavirin. This treatment is effective in 30-50% of patients and is very costly (WHO). However, recent studies have shown promising vaccine options. For example, Elmowalid *et al* demonstrated that recombinant HCV-like particles for HCV structural proteins were able to control HCV infection in chimpanzees and induce a strong cellular immune response (2007). Another study developed a multi-genotype plasmid DNA vaccine against the E1 structural protein which showed both stronger humoral and cellular immune responses (Encke *et al*, 2007). A third group has developed a peptide-based vaccine that consists of a synthetic peptide representing the HVR1 of the E2 structural protein and a sequence representing a helper T cell epitope. This vaccine was able to elicit biologically active neutralizing antibodies against homologous HCV E2 sequences (Torresi *et al*, 2007).

Vaccine Description

We have designed a peptide vaccine SISOR-P3 to protect against Hepatitis C virus infection. It is a self-adjuvanting vaccine that has epitopes for inducing neutralizing antibodies as well as a cellular immune response. The B cell epitope G31 with the sequence, TTHTVGGSVARQVHSLTGLFSPGPQQK, is a consensus profile from the HVR1 region of the envelope glycoprotein, E2 of HCV. The T helper cell epitope belongs to the NS3 protein and it includes the amino acids from 1248-1261 with the sequence GYKVLVLNPSVAAT . The cytotoxic T lymphocyte (CTL) epitope also belongs to the NS3 protein and includes the amino acids 1038-1047 with the sequence GLLGCIITSL. The peptides were made self-adjuvanting by linking them to a lipid, Pam2Cys, which occurs naturally in the Mycoplasma-derived component MALP-2 (Muhlradt et al., 1997). All peptide components of the vaccine will be synthesized by chemoselective ligation using polyoxime chemistry. In order to incorporate lipid moieties into epitope constructs, Pam2Cys was assembled with 12 lysine residues (polyK-Pam2Cys) to confer solubility. The N-terminal lysine was then acryloylated to enable copolymerization of lipid and peptide to form lipidated polyepitopes (Torresi *et al*, 2007).

Hepatitis C virus is the major cause of acute hepatitis and chronic liver disease, including cirrhosis and liver cancer. It is estimated that 170 million people worldwide and more than 10% of the population in some countries are infected with HCV. Hepatitis C infection is now the leading indication for liver transplantation in countries such as the United States. Current treatment is confined to the use of the combination of pegylated interferon- α together with the antiviral drug ribavirin. However, such a treatment achieves viral clearance in only 40-50% of patients infected with genotype 1 and 70-80% in genotypes 2 and 3 (Gremion and Cerny, 2005).

Given the global disease burden and public health impact of Hepatitis C, the development of an effective vaccine is of paramount importance. Several approaches have been used to develop a HCV vaccine. Novel vaccine candidates based on molecular technology such as recombinant proteins, peptides, virus-like particles, naked DNA and recombinant viruses are being explored (Lechmann and Liang, 2000). The immunologic correlates associated with disease progression or protection are yet to be precisely defined, but recent studies suggest that induction of high-titer, long lasting and cross-reactive anti-envelope antibodies and a vigorous multispecific cellular immune response that includes both helper and cytotoxic T lymphocytes is important in the resolution of infection (Gremion and Cerny, 2005; Elliot *et al*, 2006) and thus may be necessary for an effective vaccine.

Hence we adopted a strategy incorporating combinatorial epitopes into a single immunogen that uses novel chemistry to develop a peptide vaccine SISOR-P3 with broad coverage against HCV. SISOR-P3 contains a self-adjuvanting multiepitope immunogen that produces broad cross-reactive antibodies capable of binding to HCV envelope proteins and also induce a strong cellular immune response that includes both CD4+ and CD8+ T cells which are required for viral clearance.

Peptide vaccines offer several practical advantages such as relative ease of construction and production, chemical stability and a lack of infectious potential. Peptides may also

allow better manipulation of the immune response through the use of epitopes designed for stimulating particular subsets of T cells (Purcell *et al*, 2007). Examination of the molecular ingredients of a successful immune response against HCV have revealed a tremendous amount of biochemical diversity in clinically important epitopes and the use of our peptide vaccine candidate SISOR-P3 offers a flexible and simple way to deal with much of this complexity by bypassing the requirements for antigen processing and delivery of a precise and chemically defined payload to the APC.

We hypothesized that eliciting an anti-HCV immune response based on the induction of broadly cross-reactive neutralizing antibodies, epitope-specific CD8⁺ cytotoxic T lymphocytes and CD4⁺ responses may be highly beneficial. We also reason out that it is advantageous to concentrate on well-conserved proteins using well-characterized epitopes spread over a large percentage of the population. In light of the above, we designed a peptide vaccine X that contains 3 major epitopes, a B cell epitope derived from the HVR1 region of E2 glycoprotein, a T helper cell epitope derived from the NS3 protein and a CTL epitope also derived from the NS3 protein.

The B cell epitope derived from the HVR1 region of envelope glycoprotein E2 represents a consensus sequence most commonly recognized by patient's sera and is capable of generating cross-reactive antibodies to about 77% of the natural HVR1 variants (Puntoriero *et al*, 1998). It has been shown that antibodies directed to epitopes in the viral glycoprotein E2 (including HVR1) are neutralizing (Scarselli *et al*, 1995; van Doorn *et al*, 1995; Rosa *et al*, 1996; Farci *et al*, 1996; Zibert *et al*, 1997; Hsu *et al*, 2003; Bartosch *et al*, 2003; Yu *et al*, 2004; Tarr *et al*, 2006) and evidence supporting the protective role of neutralizing antibody was highlighted by the demonstration of the presence of neutralizing antibodies in immune globulin prepared from anti-HCV serum. This immune globulin protected chimpanzees from infection with HCV genotypes 1a and 2a, strengthening the argument that neutralizing antibodies can be cross-protective. Since SISOR-P3 generates cross-reactive antibodies to 77% of HVR1 variants, it is likely that most of the strains of HCV would be recognized using this B cell epitope. Although neutralizing antibody responses are not associated with viral clearance in acute hepatitis C infection, they do have a role in controlling viral replication in patients with chronic hepatitis C (Logvinoff *et al*, 2004)

Clearance of HCV infection is associated with the development of an early broad and persistent class-I-restricted CD8⁺ CTL and CD4⁺ T cell response to a spectrum of HCV structural and nonstructural proteins (Lechner *et al*, 2000; Missale *et al*, 1996; Diepolder *et al*, 1997; Lamonaca *et al*, 1999). Although the specificity of T cell responses associated with resolution of infection is not well defined, many reports have underlined the key role of T cells specific of the non-structural protein 3. In self-limited patients, the NS3 specific CD4⁺ T cell response was reported as the strongest and most consistently detected CD4⁺ mediated responses (Bronowiki *et al*, 1997; Diepolder *et al*, 1997; Rosen *et al*, 2002). NS3 also contains numerous class I-restricted epitopes (Cerny *et al*, 1995; Kurokohchi *et al*, 1996; He *et al*, 1999) and it has been described that patients responders to IFN- α therapy display strong CTL activity specific of one of these epitopes (Vertuani *et al*, 2002). Therefore, assuming that NS3 might represent a good vaccine candidate for

the induction of protective T cell-mediated immunity, we incorporated an immunodominant CD4⁺ T-cell epitope and a CD8⁺ T-cell epitope, found within the NS3 protein, in our candidate vaccine Sisor-P3

It has been observed that a strong NS3-specific CD4⁺ T-cell response, which is associated with viral clearance in acute hepatitis C infection, is dominated by the single 14-aa epitope (aa 1248 to 1261) that we have used in our vaccine. Moreover the response can be mounted by patients with diverse HLA background due to the promiscuous nature of the epitope to bind to most common HLA-DR alleles. Also, aa 1248-1261 are completely conserved in all 33 genotype 1a, 1b, 1c, 2a and 2b sequences. Only genotype 3a shows a change at position aa 1250 from lysine to asparagine, which lies outside the putative minimal epitope aa 1251 to 1259 (Diepolder *et al*, 1997). This may imply that viral escape is unlikely to be an important factor in the regulation of the CD4⁺ T-cell response to aa 1248 to 1261, which provides an advantage to our vaccine Sisor-P3.

Virus-specific cytotoxic T lymphocytes play a critical role in the host cellular immune response against HCV (Imawari *et al*, 1999; Rehermann *et al*, 1996). CTLs might contribute to hepatocellular damage in HCV infection as a consequence of killing virus-infected cells. On the other hand, CTLs play an important role in preventing the spread of virus and in clearing virus during infection (Nelson *et al*, 1997; Mochizuki *et al*, 1997). In particular, T cells specific of the NS3 are often associated with control of viremia. In view of this, we identified an immunodominant epitope (GLL: aa 1038-1047) mapping within the NS3 protease that elicit antiviral CTLs (Martin *et al*, 2004) and incorporated the same in Sisor-P3. GLL displayed high in vitro binding capacities to soluble HLA-A2 molecules (allele with increased prevalence in many human populations) and were able to induce either CTL and/or IFN- γ producing T cells. This peptide was also capable to recall in vitro HCV-specific IFN- γ and IL-10 producing T cells from PBMC of chronically infected patients (Martin *et al*, 2004). In addition to its high immunogenic potential, the GLL peptide epitope sequence is well conserved among strains of genotype 1a, 1b and 4 and that most of the observed mutations are localized outside the MHC anchor motifs (Martin *et al*, 2004). Both its high immunogenicity and degree of conservation underlines the potential value of this peptide in our vaccine. Moreover, there are reports which suggest that linking of a Th epitope to a CTL determinant is effective in the generation of antiviral CTLs in murine and simian systems (Shirai *et al*, 1992; Yasutomi *et al*, 1996), although the role of Th cells in the maturation of CTL precursors remains poorly understood.

Though peptides can successfully induce T and B cell responses, they can do so only in the presence of potent adjuvants because of their inability to act as effective immunogens. In order to elicit an effective immune response we used lipopeptides incorporating the epitopes of CD4⁺ T cells, CD8⁺ T cells and B cells into a lipid moiety, Pam2Cys. These lipopeptides are self-adjuncting and have proven to be immunogenic in animal models and in humans and are well tolerated in these species (Brown and Jackson, 2005). This form of vaccine candidate has great benefits in terms of providing a totally synthetic and pure product that is effective when administered in the absence of any adjuvant, and is

immunogenic when delivered by a variety of routes, including application to mucosal surfaces.

The lipopeptide delivery approach provides a method of targeting dendritic cells, which are the primary antigen presenting cells particularly for viruses. It activates dendritic cells through Toll-like receptor-2 and also induces their maturation. It allows uptake of antigen for access to both class I and class II processing and presentation pathways. It stimulates B cells to promote high levels of antibody production and also possibly stimulates T cells to lower their threshold for antigen-specific activation (Brown and Jackson, 2005). Furthermore, by incorporation of epitopes for helper T cell induction, long-lived memory responses can be achieved.

Vaccine trial

Vaccine: Self-adjuvanting lipopeptide vaccine SISO-R-P3 with epitopes for B, helper and cytotoxic T cells.

Study group: Healthy human individuals were enrolled in the study. Since the CTL epitope binds to HLA-A2 molecules, only individuals positive for HLA-A2 were chosen. T helper epitope is highly promiscuous and can bind to 10 of 13 HLA-DR alleles. So this was not included in the criteria for selecting subjects.

Vaccination: Subjects were divided into groups. The vaccine was administered intranasally. The control group received 5 ml saline and the vaccinated group received the vaccine at a dose of 5 mg peptide in 5 ml saline on days 1, 21 and 42.

The intranasal route was chosen because the nasal mucosa provides a moist and highly vascularized membrane which is crucial for fast absorption of peptides into the blood stream. Moreover no local reactions have been observed in previous studies and the delivery mode was well tolerated. The vaccine also induces strong proliferation of CD4+ T cells which preferentially secrete IFN- γ compared to those induced by the subcutaneous route which secrete IL-4 (Brown et al., 2005)

Samples: Blood samples were collected at baseline, before each vaccination and 4 and 8 weeks after the last vaccination.

Safety assessments: Physical examination and vital sign checks were performed at every study visit. Safety monitoring was carried out at the study site by the clinical investigator before vaccination, 10 min and 1, 2 and 3 hour after vaccination. In addition, the participants were requested to record any adverse reaction that might have occurred during the first 24 hour post-vaccination on an individual diary card.

Vaccine efficacy

The vaccine was then tested to show its responsiveness to the immune system. Both humoral and cellular immune responses are important for the elimination of Hepatitis C virus. So we studied the B and T cell responses to HCV antigens.

Peripheral blood mononuclear cells (PBMC) preparation

PBMCs were isolated using Ficoll-Histopaque and used in assays for studying HCV-specific cellular responses.

ELISA (Enzyme linked immunosorbent assay)

ELISA was performed to determine the presence of neutralizing antibodies against the envelope glycoprotein E2 of HCV. E2 was used as the antigen and was coated on the microtiter plate. Dilutes serum samples from patients immunized with SISOR-P3 was then added to the plate and incubated. Anti human-IgG antibodies tagged with the enzyme were then added and incubated. Binding of the antigen to the antibody was determined by the conversion of a colorless substrate to a colored reaction product which indicated the presence of neutralizing antibodies.

Assay for cytotoxic T cells

HCV-specific CD8⁺ cells were determined by Chromium release assay. Target cells were labeled with radioactive chromium and then PBMCs isolated from vaccinated individuals were added. In the presence of HCV-specific CD8⁺ cytotoxic T cells, the target cells will be lysed and the extracellular chromium released will be measured using a radioactive counter.

ELISPOT

This test was performed to determine the IFN- γ levels produced by HCV-specific CD4⁺ and CD8⁺ T cells. PBMCs from vaccinated individuals were incubated with the peptide followed by incubation with anti-human IFN- γ mAb (tagged with enzyme) coated on a microtiter plate. HCV-specific activated T cells secreting IFN- γ will be captured by the antibody. Secondary antibody tagged with substrate was then added to reveal a circle of bound cytokine surrounding the position of each activated T cell. Thus counting each spot tell us the frequency of the T cells producing the cytokine.

T cell Proliferation assay

The T cell proliferation assay was use to analyze HCV peptide specific T helper cell responses using thymidine incorporation assay. PBMCs from vaccinated individuals were cultivated along with the peptides for 6 days. [³H]Thymidine was added to each well and the cells were then cultured and harvested. Incorporated radioactivity was measured using a liquid scintillation beta counter.

HLA-A *0201 tetramer- binding assay (FACS)

This assay was used to determine presence of HCV-specific CD4⁺ and CD8⁺ memory T cells. PBMCs were stained with antibodies against CD3, CD4/CD8 and CD45RO (memory T cell marker) together with an HLA-tetramer specific for T cells specific for respective peptide epitope. The tetrameric complexes were then analyzed by flow cytometry.

Results

The following criteria will be used as indicators of vaccine efficacy:

Vaccinated individuals should not experience any vaccine related adverse effects. Neutralizing antibodies should be generated and must have undergone seroconversion. Effective HCV-specific CD4+ T cell and CD8+ T cell responses should be generated. All results must be statistically significant from the control groups.

Problems encountered and strategies adopted to overcome them

Hepatitis C virus exhibits high frequency of antigenic variation. There are no clearly defined antigens which can be targeted to generate a protective immune response. Hence it is more appropriate to use individual epitopes for eliciting an effective immune response. Moreover due to the existence of several genotypes and subtypes, a vaccine that has a broad reactivity is essential. By using individual epitopes for activating both the humoral and cellular arms of the immune system as well as by incorporating sequences which are conserved among different subtypes, we have overcome this limitation.

The intranasal route of administration limits the maximum dose of peptide per spray and also tends to get rapidly cleared from the nasal cavity. So in order to elicit a more effective response, we have recommended a higher dosage of the vaccine and also the requirement for two boosters.

Reference:

Anzola M, Burgos JJ. Hepatitis C virus (HCV): model structure and genome organization. **Expert Reviews in Molecular Medicine**, **5**: 1, 2003.

Bartosch B, Bukh J, Meunier JC, Granier C, Engle RE, Blackwelder WC, Emerson SU, Cosset FL, Purcell RH. In vitro assay for neutralizing antibody to hepatitis C virus: evidence for broadly conserved neutralization epitopes. **Proc. Natl. Acad. Sci.**, **100**:14199-14204, 2003.

Bronowicki JP, Vetter D, Uhl G, Hudziak H, Uhrmacher A, Vetter JM, Doffoel M. Lymphocyte reactivity to hepatitis C virus (HCV) antigens shows evidence for exposure to HCV in HCV-seronegative spouses of HCV-infected patients. **J. Infect. Dis.**, **176**(2):518-522, 1997.

Brown LE, Jackson DC. Lipid-based self-adjuvanting vaccines. **Curr. Drug Deliv.**, **2**(4):383-393, 2005.

Cavanaugh VJ, Guidotti LG, Chisari FV. Interleukin-12 inhibits hepatitis B virus replication in transgenic mice. **J. Virol**, **71**: 3236-3243, 1997.

Cerny A, McHutchison JG, Pasquinelli C, Brown ME, Brothers MA, Grabscheid B, Fowler P, Houghton M, Chisari FV. Cytotoxic T lymphocyte response to hepatitis C

virus-derived peptides containing the HLA A2.1 binding motif. **J. Clin. Invest.**, **95(2)**:521-530, 1995.

Cheryl LD, George ML, Gregory KR, McGovern B. Broad specificity of Virus specific CD4+ T-Helper cell responses in resolved Hepatitis C virus infection. **Journal of Virology**, **76**:12584-12595, 2002.

Diepolder HM, Gerlach JT, Zachoval R, Hoffmann RM, Jung MC, Wierenga EA, Scholz S, Santantonio T, Houghton M, Southwood S, Sette A, Pape GR. Immunodominant CD4(+) T-cell epitope within nonstructural protein 3 in acute hepatitis C virus infection. **J. Virol.**, **71**:6011-6019, 1997.

Elliot LN, Lloyd AR, Ziegler JB, French RA. Protective immunity against hepatitis C virus infection. **Immunol. Cell Biol.**, **84(3)**:239-249, 2006.

Elmowalid GA, Qiao M, Jeong S, Borg BB, Baumert TF, Sapp RK, Hu Z, Murthy K, Liang TJ. Immunization with hepatitis C virus-like particles results in control of hepatitis C virus infection in chimpanzees. **Proc. Natl. Acad. Sci.**, **104**: 8427-8432, 2007.

Encke J, Radunz W, Eisenbach C, Geib J, Gehrke S, Pfaff E, Stremmel W. Development of a heterologous, multigenotype vaccine against hepatitis C virus infection. **European Journal of Clinical Investigation**, **37**: 396-406, 2007.

Farci P, Shimoda A, Wong D, Cabezon T, De Gioannis D, Strazzer A, Shimizu Y, Shapiro M, Alter HJ, Purcell RH. Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. **Proc. Natl. Acad. Sci.**, **93**:15394-15399, 1996.

Gremion C, Cerny A. Hepatitis C virus and the immune system: a concise review. **Rev. Med. Virol.**, **15(4)**:235-268, 2005.

He XS, Rehmann B, Lopez-Labrador FX, Boisvert J, Cheung R, Mumm J, Wedemeyer H, Berenguer M, Wright TL, Davis MM, Greenberg HB. Quantitative analysis of hepatitis C virus-specific CD8+ T cells in peripheral blood and liver using peptide-MHC tetramers. **Proc. Natl. Acad. Sci.**, **96(10)**:5692-5697, 1999.

Hsu M, Zhang J, Flint M, Logvinoff C, Cheng-Mayer C, Rice CM, McKeating JA. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. **Proc. Natl. Acad. Sci.**, **100**:7271- 7276, 2003.

Imawari M, Nomura M, Kaieda T, Moriyama T, Oshimi K, Nakamura I, Gunji T, Ohnishi S, Ishikawa T, Nakagama H, Takaku F. Establishment of a Human T-Cell Clone Cytotoxic for Both Autologous and Allogeneic Hepatocytes from Chronic Hepatitis Patients with Type Non-A, Non-B Virus. **Proc. Natl. Acad. Sci.**, **86(8)**:2883-2887, 1989.

Keck Z, Vicky MH, Susan P, Judy R, Paul S, Liang TJ, Lai MMC, Fong SKH. Human Monoclonal Antibody to Hepatitis C Virus E1 Glycoprotein That Blocks Virus Attachment and Viral Infectivity. **Journal of virology**, **78**:7257-7263, 2004.

Kurokohchi K, Akatsuka T, Pendleton CD, Takamizawa A, Nishioka M, Battagay M, Feinstone SM, Berzofsky JA. Use of recombinant protein to identify a motif-negative human cytotoxic T-cell epitope presented by HLA-A2 in the hepatitis C virus NS3 region. **J. Virol.**, **70(1)**:232-240, 1996.

Lamonaca V, Missale G, Urbani S, Pilli M, Boni C, Mori C, Sette A, Massari M, Southwood S, Bertoni R, Valli A, Fiaccadori F, Ferrari C. Conserved hepatitis C virus sequences are highly immunogenic for CD4(+) T cells: implications for vaccine development. **Hepatology**, **30**:1088-1098, 1999.

Lau JY. Mechanisms of Hepatic Toxicity IV: Pathogenic mechanisms involved in hepatitis C virus-induced liver disease. **Am J Physiol Gastrointest Liver Physiol.**, **275**:G1217-G1220, 1998.

Lechmann M, Liang TJ. Vaccine development for hepatitis C. **Semin. Liver Dis.**, **20(2)**:211-226, 2000.

Lechner F, Wong DK, Dunbar PR, Chapman R, Chung RT, Dohrenwend P, Robbins G, Phillips R, Klenerman P, Walker BD. Analysis of successful immune responses in persons infected with hepatitis C virus. **J. Exp. Med.**, **191**:1499-1512, 2000.

Li K, Foy E, Ferreon JC, Nakamura M, Ferreon AC, Ikeda M, Ray SC, Gale M Jr, Lemon SM. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. **Proc. Nat. Acad. Sci. USA**, **102**:2992-2997, 2005.

Logvinoff C, Major ME, Oldach D, Heyward S, Talal A, Balfe P, Feinstone SM, Alter H, Rice CM, McKeating JA. Neutralising antibody response during acute and chronic hepatitis C virus infection. **Proc. Natl. Acad. Sci.**, **101**:10148-10154, 2004.

Martin P, Parroche P, Chatel L, Barretto C, Beck A, Trepo C, Bain C, Lone YC, Inchauspe G, Fournillier A. Genetic immunization and comprehensive screening approaches in HLA-A2 transgenic mice lead to the identification of three novel epitopes in hepatitis C virus NS3 antigen. **J. Med. Virol.**, **74(3)**:397-405, 2004.

Missale G, Bertoni R, Lamonaca V, Valli A, Massari M, Mori C, Rumi MG, Houghton M, Fiaccadori F, Ferrari C. Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. **J. Clin. Invest.**, **98**:706-714, 1996.

Mochizuki K, Hayashi N, Katayama K, Hiramatsu N, Kanto T, Mita E, Tatsumi T, Kuzushita N, Kasahara A, Fusamoto H, Yokochi T, Kamada T. B7/BB-1 expression and

hepatitis activity in liver tissues of patients with chronic hepatitis C. **Hepatology**, **25(3)**:713-718, 1997.

Moriishi K, Matsuura Y. Mechanisms of Hepatitis C Virus Infection. **Antivir Chem Chemother.**, **6**: 285-297, 2003.

Mühlradt PF, Kiess M, Meyer H, Sussmuth R, Jung G. Isolation, Structure Elucidation, and Synthesis of a Macrophage Stimulatory Lipopeptide from *Mycoplasma fermentans* Acting at Picomolar Concentration. **J. Exp. Med.**, **185**:1951-1958, 1997.

Nelson DR, Marousis CG, Davis GL, Rice CM, Wong J, Houghton M, Lau JY. The role of hepatitis C virus specific cytotoxic T lymphocytes in chronic hepatitis C. **J. Immunol.**, **158(3)**:1473-1481, 1997.

Netski DM, Mosbrugger T, Depla E, Maertens G, Ray SC. Humoral Immune Response in Acute Hepatitis C Virus Infection. **Clinical Infection Disease**, **41**: 667-675, 2005.

Puntoriero G, Meola A, Lahm A, Zucchelli S, Ercole BB, Tafi R, Pezzanera M, Mondelli MU, Cortese R, Tramontano A, Galfre' G, Nicosia A. Towards a solution for hepatitis C virus hypervariability: mimotopes of the hypervariable region 1 can induce antibodies cross-reacting with a large number of viral variants. **EMBO, J.** **17**:3521-3533, 1998.

Purcell AW, McCluskey J, Rossjohn J. More than one reason to rethink the use of peptides in vaccine design. **Nat. Rev. Drug. Discov.**, **6(5)**:404-414, 2007.

Rehermann B, Chang KM, McHutchinson J, Kokka R, Houghton M, Rice CM, Chisari FV. Differential cytotoxic T lymphocyte responsiveness to the hepatitis B and C viruses in chronically infected patients. **J. Virol.**, **70(10)**:7092-7102, 1996.

Rosa D, Campagnoli S, Moretto C, Guenzi E, Cousens L, Chin M, Dong C, Weiner AJ, Lau JYN, Choo Q, Chien D, Pileri P, Houghton M, Abrignani S. A quantitative test to estimate neutralizing antibodies to the Hepatitis C virus: Cytofluorimetric assessment of envelope glycoprotein 2 binding to target cells. **Proc. Nat. Acad. Sci. USA**, **93**:1759-1763, 1996.

Rosen HR, Hinrichs DJ, Glenda R. Cutting Edge: Identification of Hepatitis C Virus-Specific CD8 T cells Restricted by Donor HLA Alleles following Liver Transplantation. **Journal of immunology**, **173**:5355-5359, 2004.

Rosen HR, Miner C, Sasaki AW, Lewinsohn DM, Conrad AJ, Bakke A, Bouwer HG, Hinrichs DJ. Determination of Hepatitis C virus-specific effector CD4+ T cell by flow cytometry: Correlation with clinical disease stages. **Hepatology**, **35(1)**:190-198, 2002.

Rustgi VK. The epidemiology of hepatitis C infection in the United States. **J. Gastroenterol.**, **42**: 513-521, 2007.

Scarselli E, Cerino A, Esposito G, Silini E, Mondelli MU, Traboni C. Occurrence of antibodies reactive with more than one variant of the putative envelope glycoprotein (gp70) hypervariable region 1 in viremic hepatitis C virus-infected patients. **J. Virol.**, **69**:4407-4412, 1995.

Shirai M, Akatsuka T, Pendleton CD, Houghten R, Wychowski C, Mihalik K, Feinstone S, Berzofsky JA. Induction of cytotoxic T cells to a cross-reactive epitope in the hepatitis C virus nonstructural RNA polymerase-like protein. **J. Virol.**, **66**:4098-4106, 1992.

Sugimoto K, Ikeda F, Stadanlick J, Nunes FA, Alter HJ, Chang KM. Suppression of HCV-specific T cells without differential hierarchy demonstrated *ex vivo* in persistent HCV infection. **Hepatology**, **38**: 1437-1448, 2003.

Tarr AW, Owsianka AM, Timms JM, McClure CP, Brown RJ, Hickling TP, Pietschmann T, Bartenschlager R, Patel AH, Ball JK. Characterization of the Hepatitis C Virus E2 epitope defined by the broadly neutralizing monoclonal antibody AP33. **Hepatology**, **43**:592-601, 2006.

Tasi SL, Liaw YF, Chen MH, Huang CY, Kuo GC. Detection of type 2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. **Hepatology**, **25**: 449-458, 1997.

Thomson M, Nascimbeni M, Havert MB. The clearance of hepatitis C virus infection in chimpanzees may not necessarily correlate with the appearance of acquired immunity. **J. Virol.**, **77**: 862-877, 2003.

Torresi J, Stock OM, Fischer AE, Grollo L, Drummer H, Boo I, Zeng W, Earnest-Silveira L, Jackson DC. A self-adjuvanting multiepitope immunogen that induces a broadly cross-reactive antibody to hepatitis C virus. **Hepatology**, **45**:911-920, 2007.

van Doorn LJ, Capriles I, Maertens G, DeLeys R, Murray K, Kos T, Schellekens H, Quint W. Sequence evolution of the hypervariable region in the putative envelope region E2/NS1 of hepatitis C virus is correlated with specific humoral immune responses. **J. Virol.**, **69(2)**:773-778, 1995.

Vertuani S, Bazzaro M, Gualandi G, Micheletti F, Marastoni M, Fortini C, Canella A, Marino M, Tomatis R, Traniello S, Gavioli R. Effect of interferon-alpha therapy on epitope-specific cytotoxic T lymphocyte responses in hepatitis C virus-infected individuals. **Eur. J. Immunol.** **32(1)**:144-154, 2002.

Wedmeryer H, He, X, Reherrmann, B. Impaired Effector Function of Hepatitis C Virus – Specific CD8 Tcells in Chronic Hepatitis C virus Infection. **Journal of Immunology**, **169**:3447-3458, 2002.

Wikipedia. Hepatitis C. Accessed: 2007. http://en.wikipedia.org/wiki/Hepatitis_C

World Health Organization. Hepatitis C. WHO 2007.
http://www.who.int/immunization/topics/hepatitis_c/en/index.html

Worman, H.J. *The Hepatitis C Sourcebook*. McGraw-Hill, New York, 2002.

Yasutomi Y, Robinson HL, Lu S, Mustafa F, Lekutis C, Arthos J, Mullins JI, Voss G, Manson K, Wyand M, Letvin NL. Simian immunodeficiency virus-specific cytotoxic T-lymphocyte induction through DNA vaccination of rhesus monkeys. **J. Virol.**, **70(1)**:678-681, 1996.

Yu MY, Bartosch B, Zhang P, Guo ZP, Renzi PM, Shen LM, Granier C, Feinstone SM, Cosset FL, Purcell RH. Neutralising antibodies to hepatitis C virus (HCV) in immune globulins derived from anti-HCV-positive plasma. **Proc. Natl. Acad. Sci.**, **101**: 7705-7710, 2004.

Zibert A, Kraas W, Meisel H, Jung G, Roggendorf M. Epitope mapping of antibodies directed against hypervariable region 1 in acute self-limiting and chronic infections due to hepatitis C virus. **J. Virol.**, **71**:4123-4127, 1997.