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DEVELOPMENT OF VACCINES AND EXPERIMENTAL MODELS FOR CHRONIC INFECTIONS CAUSED BY THE HEPATITIS C VIRUS

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Cover picture: Detection of the hepatitis C virus NS3 protein expression in a 4μm thick liver section from an NS3/4A-transgenic mouse using a human anti-NS3 antibody.

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To my family

SUMMARY

The hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. It is estimated that HCV affects approximately 170 million people around the world. One feature of HCV infection is the high rate of viral persistence. The mechanism of viral persistence is largely unknown, although the high genetic variability is thought to play a key role. Today, no vaccine is available to prevent or cure HCV infections, albeit antiviral therapy is used quite effectively. This study aimed at developing new vaccines and new model systems to study HCV. We studied the HCV NS3 protein in detail since it performs key functions in the viral life cycle. These are unwinding and strand separation of the viral RNA and proteolytic processing of the precursor polyprotein. To obtain the complete protease we included the NS4A co-factor in our NS3-based vaccines. NS4A has been shown to enhance the stability of NS3 and to target the NS3/4A complex to intracellular membranes. The latter is most likely of importance for the formation of the replication complex. Also, the NS3 region has a limited genetic variability and several studies have now demonstrated that NS3-specific CD4+ and CD8+ T-cell responses are crucial for the resolution of HCV infections. Thus, several factors suggest that the NS3 region should be well suited for vaccine development.

We could show that HCV NS3-based genetic vaccines effectively primed both humoral and cellular immune responses in mice. NS3/4A was shown to prime a Th1 CD4+ T-cell responses. The inclusion of NS4A in NS3-based vaccines primed antibody, CD4+, and CD8+ T-cell responses that were superior to those primed by NS3-gene alone. Thus, NS4A enhanced the immunogenicity of NS3. We could show that enhancement of the immunogenicity was most probably a result of the higher expression levels of NS3 generated by the inclusion of NS4A. We next tested if the overall immunogenicity of NS3/4A could be further enhanced by codon optimization or by mRNA amplification using the Semliki forest virus (SFV) replicon. The NS3 protein expression levels were further improved by either codon optimization and mRNA amplification. Subsequently, both these modifications enhanced the NS3-specific immune responses. One concern in development of genetic vaccines is that the gene displays unwanted properties when expressed *in vivo*. We therefore, generated a new transgenic mouse expressing the HCV NS3/4A-protein in the liver. The protein expression was restricted to the liver to mimic the *in vivo* situation during a HCV infection. Protein expression was localized to the cytoplasm of the hepatocytes and displayed a similar staining pattern as seen in hepatocytes from HCV infected individuals. The intra-hepatic protein expression did not cause overt liver damage, except for a slight enlargement of the liver. However, the NS3/4A-transgenic mice displayed less spontaneously appearing intra-hepatic inflammatory foci, which are commonly found in laboratory mice. Thus, expression of NS3/4A-protein may affect the distribution of immune cells within the liver.

The present studies demonstrate that NS3-based genetic vaccines that contain NS4A more effectively prime humoral and cellular immune responses against NS3. Intra-hepatic expression of NS3/4A did not cause any spontaneous liver disease or overt pathology suggesting that it safely can be used in genetic vaccines. Thus, the NS3/4A gene can safely activate immune responses that are similar to those found in humans who can clear HCV. The NS3/4A should therefore be a potential vaccine candidate against chronic HCV infections.

Key words: HCV, chronic infection, DNA vaccine, NS3, viral vectors, transgenic mice

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LIST OF PUBLICATIONS

The thesis is based on the following papers, which will be referred to by their Roman numerals (I-IV):

I. Lazdina U, Hultgren C, **Frelin L**, Chen M, Lodin K, Weiland O, Leroux-Roels G, Quiroga J.A, Peterson D.L, Milich D.R and Sällberg M. Humoral and CD4+ T helper (Th) cell responses to the hepatitis C virus non-structural 3 (NS3) protein: NS3 primes Th1-like responses more effectively as a DNA-based immunogen than as a recombinant protein. Journal of General Virology (2001), 82, 1299-1308.

II. **Frelin L**, Alheim M, Chen A, Söderholm J, Rozell B, Barnfield C, Liljeström P, and Sällberg M. Low dose and gene gun immunization with a hepatitis C virus nonstructural (NS) 3 DNA based vaccine containing NS4A inhibit NS3/4A-expressing tumors *in vivo*. Gene Therapy (2003), 10, 686-699.

III. **Frelin L**, Ahlén G, Alheim M, Weiland O, Barnfield C, Liljeström P, and Sällberg M. Codon optimization and mRNA amplification effectively enhances the immunogenicity of the hepatitis C virus (HCV) nonstructural (NS) 3/4A gene. Gene Therapy (2004), 11, 522-533.

IV. **Frelin L**, Glaumann H, Rozell B, and Sällberg M. Intra-hepatic expression of the hepatitis C virus (HCV) non-structural (NS) 3/4A protein complex in transgenic mice. (*Manuscript*).

CONTENTS

LIST OF ABBREVIATIONS

aa	amino acid
APC	Antigen presenting cell
ALT	Alanine aminotransferase
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immuno sorbent assay
ER	Endoplasmic reticulum
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HEV	Hepatitis E virus
HCC	Hepatocellular carcinoma
HVR	Hyper variable region
IFN-α	Interferon-alpha
IFN-γ	Interferon-gamma
IL	Interleukin
MHC	Major histocompatibility complex
NK	Natural killer cell
NKT	Natural killer T cell
NS	Non-structural
ORF	Open reading frame
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SFV	Semliki forest virus
Th	T-helper
TNF-α	Tumor necrosis factor alpha

INTRODUCTION

Viral hepatitis is one of the major public health problems around the world and it affects several hundreds of millions of people. Viral hepatitis can cause both acute and chronic infections, which is one of the most common causes of morbidity and mortality worldwide. Today, there are five human viruses that are primarily hepatotropic, the hepatitis A, B, C, D, and E viruses. These viruses account for approximately 90% of all acute viral hepatitis and 95% of all chronic viral hepatitis. Other viral infections also associated with hepatitis are the Epstein-Barr virus (EBV), cytomegalovirus (CMV), herpes simplex virus (HSV), and varicella zoster virus (VZV).

Once a person gets infected with a hepatitis virus, the virus is spread by the blood stream to the liver, the target organ. The virus will then cause an acute infection that over time may progress to a chronic infection. Virus particles infect the liver cells and directly or indirectly cause destruction of the hepatocytes. When liver cells die, intracellular enzymes such as transaminases are released. These enzymes can easily be detected in the serum of infected patients during both acute and chronic infections. The acute infection can be either asymptomatic or symptomatic. Acute symptomatic hepatitis, or what is often referred to as the icteric phase of the infection, is characterized by jaundice and elevated liver transaminases. Symptoms in the preicteric phase are myalgia, nausea, vomiting, fatigue and malaise. Impaired liver function is associated with accumulation of substances in the body that are metabolized in the liver. Before the development of good diagnostic tools chronic infections caused by hepatitis viruses were defined by abnormal liver enzyme values persisting for more than 6 months. Chronic viral hepatitis is characterized by the presence of inflammatory infiltrates in the liver associated with hepatocyte death. The majority of the patients with chronic viral hepatitis are, however, asymptomatic. Chronic infections are associated with a more severe clinical outcome, such as liver cirrhosis (liver scarring) and hepatocellular carcinoma (HCC; liver cancer) after 10-30 years of infection.

HEPATITIS VIRUSES

Hepatitis A virus

Hepatitis A virus (HAV) belongs to the Picornaviridae virus family and is divided into the genus Hepatovirus. HAV is a RNA virus that was discovered in 1973 [1] and it is transmitted fecal-orally with an incubation time of 2-6 weeks. The infection is acute with various clinical outcomes, ranging from subclinical, mild illness in young children to the full range of symptoms with jaundice in adults. HAV is diagnosed by detection of specific IgM antibodies. Prevention is possible by passive immunization using anti-HAV immunoglobulin or active immunization using the hepatitis A vaccines. Vaccination is recommended to risk groups and to people going to highly endemic areas. Today, there is a lack of antiviral treatment against HAV.

Hepatitis B virus

Hepatitis B virus (HBV) is a member of the Hepadnaviridae family and further divided into the Orthohepadnavirus genus. HBV, or more correctly the surface antigen of HBV (HBsAg, initially termed the Australia-, or Au-, antigen), was discovered in 1965 [2] and the HBV virion was visualized in 1970 [3]. The virus has a partially double stranded circular DNA genome. One characteristics of the virus is that the mature DNA genome is generated from an RNA pre-genome through reverse transcription. The virus is endemic in the human population and is transmitted through contaminated blood, from mother to child, and by sexual contacts. The virus incubation period is 2-6 months. Approximately 350 million people worldwide are carriers of HBV and it is estimated that 1 to 2 million people die annually due to the infection. HBV causes both acute and chronic infections. The virus can establish a persistent infection in approximately 5-10% of those infected as adults and in as many as 90% of those infected perinatelly. The cellular immune responses directed against the infected hepatocytes are believed to mediate the pathology. Long-term viral replication may lead to progression to chronic liver disease, cirrhosis and HCC. During viral replication the HBV genome may integrate into the chromosomal DNA of the infected hepatocytes. Acute HBV infection is diagnosed by the simultaneous detection of IgM antibodies specific for the HBV core

antigen (HBcAg) and HBsAg. The clinical features of acute infection is anicteric and asymptomatic, although a severe illness with jaundice and acute liver failure may develop. Chronic HBV infection is diagnosed by detection of HBsAg and lack of anti-HBc IgM. Prevention is possible by passive immunization using immunoglobulin specific for HBV or by active immunization. Currently used treatments for chronic HBV infections include interferon-α (IFN-α) and lamivudine.

Hepatitis C virus

Hepatitis C virus (HCV) will be described in detail below.

Hepatitis D virus

Hepatitis D virus (HDV) is a replicative defective virus, which requires the presence of HBsAg for infectivity. HDV was discovered 1977 [4] and is transmitted through blood-blood contacts. The virus has an incubation period of 2-6 weeks and the clinical features are similar to those of hepatitis A virus. HDV is unclassified, but show similarity with certain satellite virus and viroids. HDV infections occur only in patients infected with HBV previously (super infection) or simultaneously (co-infection). This is due to the fact that HDV is an incomplete RNA virus that needs to use the HBsAg from HBV for infectivity.

HDV causes both acute and chronic infections and it is estimated that approximately 5% of all HBsAg carriers worldwide (around 18 million people) are infected with HDV. Super infection with HDV is known to cause a more severe and rapid progression of liver disease, due to the presence of large amount of HBsAg, which allows for a rapid replication of HDV. HDV is diagnosed by detection of anti-HDV IgM, HDV RNA or HDAg in serum. Prevention against HBV/HDV co-infection is possible by vaccination against HBV, since HDV is dependent on HBV for its replication. The major problem is to protect against super infection of the many millions of chronic HBV carriers. Vaccination is recommended to risk groups and the current available antiviral treatment is the same as for HBV.

Hepatitis E virus

Hepatitis E virus (HEV) is transmitted fecal-orally and was discovered in 1983 in the feces from an experimentally infected human volunteer [5]. The clinical outcome of a HEV infection is similar to HAV with an incubation time of 4-8 weeks. The virus is at present not classified but may belong to either the Caliciviridae or the Togaviridae. HEV is diagnosed by detection of anti-HEV IgM and HEV RNA. Prevention is possible by using safe water supplies, safe disposal of feces and good personal hygiene. Today, there is no available preventive vaccine or antiviral drugs.

The characteristics, viral families and transmission of hepatitis viruses are summarized in table 1.

Virus	Characteristics	Viral family / genus	Mode of transmission	Acute / chronic infection
HAV	+ssRNA, linear genome, icosahedral capsid, non-enveloped	Picornaviridae / Hepatovirus	Fecal-oral	Acute
HBV	dsDNA with an RNA pre-genome, icosahedral capsid, enveloped	Hepadnaviridae / Orthohepadnavirus	Blood-blood, sexual	Acute / chronic
HCV	+ssRNA, linear genome, icosahedral capsid, enveloped	Flaviviridae / Hepacivirus	Blood-blood	Acute / chronic
HDV	circular ssRNA, requires the presence of HBV to replicate	Related to satellite virus and viroids (unclassified)	Blood-blood	Acute / chronic
HEV	+ssRNA, un-segmented genome, icosahedral capsid, non-enveloped	Caliciviridae or Togaviridae	Fecal-oral	Acute

Table 1. The hepatitis viruses.

HEPATITIS C VIRUS

History

Hepatitis C virus (HCV) is a Hepacivirus belonging to the Flaviviridae family [6]. The genome of HCV is organized in a manner similar to that of the *flaviviruses* (e.g., yellow fever virus (YFV)) and *pestiviruses* (e.g., bovine viral diarrhea virus (BVDV)) [7]. HCV was discovered in 1989 using molecular techniques from an experimentally infected chimpanzee and at the same time the first diagnostic test was described [8, 9]. The virus is spread mainly through blood-blood contacts, and it is estimated that approximately 3% of the world population is chronically infected with HCV. Since the introduction of screening of blood products in 1990 - 1991, intravenous drug use has become the almost exclusive mode of HCV transmission in northern Europe and North America. Before screening of blood products HCV turned out to be the major cause of transfusion transmitted non-A-non-B hepatitis.

The HCV genome

The infectious HCV particle is approximately 30-60nm in diameter. The virion has a spherical shape and is bounded by a lipid-containing envelope, consisting of the two structural glycoproteins (envelope 1 and 2 proteins; E1 and E2). The genome of HCV consists of a positive sense, single-stranded linear RNA molecule, containing approximately 9,6 kilo bases, encoding a single large open reading frame (ORF) extending throughout most of its length [8, 10-12]. This large ORF encodes a single polyprotein of 3010 to 3033 aminoacids (aas) and contains at least ten viral proteins: NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH [13, 14]. The single large precursor polyprotein is processed co- and post-translationally into individual structural (C, E1, E2) and non-structural (NS2, NS3, NS4A, NS4B, NS5A, NS5B) viral proteins by a combination of host signalases and viral proteases (Figure 1). Today it is not known if p7 is structural or non-structural protein.

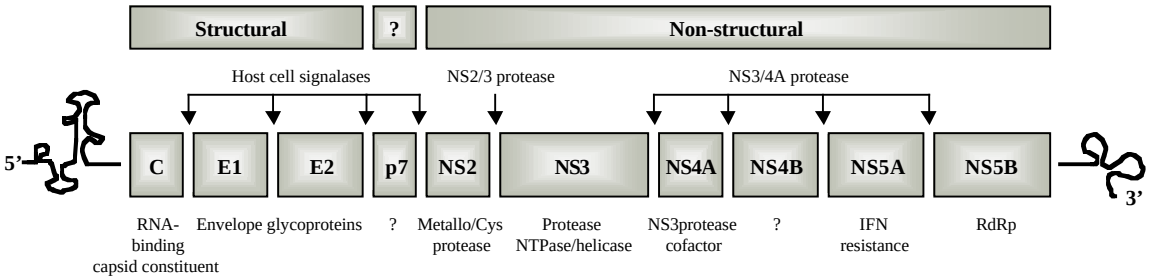

Figure 1. Schematic illustration of HCV genomic organization, polyprotein processing and protein properties.

Processing of the structural proteins located in the amino terminal quarter of the polyprotein is mediated by host signalases cleaving in the lumen of the endoplasmatic reticulum (ER) generates the putative nucleocapsid protein (core) and the envelope glycoproteins E1 and E2 [15-18]. The core protein is a basic RNA-binding phosphoprotein and probably the major constituent of the viral nucleocapsid. In addition, the core protein also appears to be involved in the modulation of several cellular processes, as the modulation of the transcription of genes that regulate cellular proliferation [19-24]. Both envelope proteins are heavily glycosylated transmembrane proteins that are located outside the core and anchored into the lipid bilayer derived from the host cell. The E2 protein contains two hyper variable regions (HVR1 and HVR2), which shows the highest variability within the HCV genome. The smallest of the HCV proteins, p7 is hydrophobic and it is not known whether p7 is a structural protein or have some other function in virus replication. Lohmann *et al* demonstrated that p7 is not critical for RNA replication [25]. However, a recent report showed that p7 protein is essential for HCV infectivity in a chimpanzee model [26]. It has also been postulated that p7 may function as a virus-encoded ion-channel [27]. The structural proteins are all included in the virion, while the nonstructural proteins are essential for RNA replication, polyprotein processing and assembly of the virus. Maturation of the NS proteins is mediated by two virally encoded enzymes: the NS2/3 protease, a zinc-dependent metallo/cysteine protease cleaving the junction between NS2 and NS3 [28, 29], and a chymotrypsin-like serine-type protease located in the amino terminal 180 amino acids of NS3 which is essential for cleaving at NS3/4A, NS4A/B, NS4B/5A, and NS5A/B sites [28, 30-34]. In the remainder of the NS3, nucleoside triphosphatase (NTPase)/RNA helicase activity has been found [13]. The NS3 helicase activity seems to play an important role in the life cycle of HCV, since the enzyme has the ability to unwind

double-stranded regions of DNA or RNA in an NTP (usually ATP)-dependent manner. The intracellular part of the life cycle of HCV starts with the release of the viral RNA in the cell cytoplasm. The HCV genome has a 5′-ribosome binding RNA structure by which the genome attaches to ribosomes and translation of the polyprotein starts. In order for HCV to replicate, negative-stranded RNA must be synthesized using the incoming positive-stranded RNA as a template. The enzyme responsible for this is the RNA-dependent RNA polymerase, or NS5B. The negative-stranded replicative intermediate is then used as a template to synthesize positive-stranded progeny RNA, which is packaged into viral capsids and used as a template for protein synthesis. Because the positive and negative RNA strands are complementary, NS3 helicase is thought to be required for unwinding and strand separation of the + and – stranded RNA. NS4A is a 54 aa cofactor of the NS3 protease and essential for its proteolytic activity [35-37]. In addition to serving as a protease cofactor, NS4A has two further functions that might contribute to efficient polyprotein cleavage and replication. First, increasing the metabolic stability of NS3 that in the absence of NS4A is degraded very rapidly, and second, anchoring NS3 to intracellular membranes where most of the HCV proteins are located, thereby increasing the local enzyme-substrate concentration and facilitating the formation of a membrane-associated replicase complex [38, 39]. When expressing NS3 alone, it is diffusely distributed in the cytoplasm and nucleus. In contrast, when expressed together with NS4A, NS3 is found in association with the ER membranes. Deletion analysis clearly visualized that it is the hydrophobic N-terminal domain of NS4A that was required for ER targeting of NS3 [39]. It has also been reported that RNA unwinding activities of the NS3 helicase is enhanced by the presence of the NS3 protease and NS4A domain [40]. The enhancement, may be due to a stabilization of the helicase fold in the full-length complex [41] and due to RNA binding sites in the protease domain contributing to helicase substrate binding [42]. NS3 and NS4A have recently been shown to interfere with the IFN signaling pathway by blocking the phosphorylation and effector function of interferon regulatory factor-3 (IRF-3), which may contribute to immune escape and viral persistence [43]. Virtually nothing is known about NS4B except its hydrophobic nature and its localization to the ER membrane [44]. NS5A is a phosphoprotein and presumably a component of the replicase complex, although its function in RNA multiplication remains to be determined. It has been suggested that NS5A plays a role in regulating HCV replication, since a high mutation rate within one region of NS5A, called interferon sensitivity-determining region (ISDR) is associated with low viral RNA titers [45, 46]. NS5A also appears to be involved

in IFN resistance [47-51]. The most C-terminal cleavage product of the viral polyprotein is NS5B, the RNA-dependent RNA polymerase (RdRp). The HCV ORF is also flanked at the 5' and 3' ends by un-translated regions (UTRs): the 5' UTR and the 3' UTR. The 5' UTR is relatively long (341nt) and it is highly conserved among all genotypes [52, 53]. The 5' UTR functions as an internal ribosome entry site (IRES) and is required for efficient translation of the polyprotein [54]. Contrary to the 5' UTR, the sequence of the 3' UTR consists of a short sequence (approximately 40 nt), which was largely diversified among different genotypes, and a homopolymer tail of A [55] or U residues [10, 11, 56, 57]. The poly A/U tail is then followed by a highly conserved 98 nt sequence, which may be important for the initiation of minus strand synthesis during RNA replication.

Viral life cycle

Studies of HCV have been limited due to the lack of convenient infectious animal models and reliable cell culture systems for virus propagation. Thus, our current understanding of the molecular mechanisms of HCV replication is based on the current available *in vivo* data generated from infectious experiments in chimpanzees and on analogies to the closely related flavi- and pestiviruses and on the characterization of recombinant HCV proteins. HCV replication cycle can be summarized as follows: 1) penetration of the host cell and un-coating; 2) release of genomic RNA from the virus particle to the cytoplasm; 3) translation of positive-strand RNA, processing of the polyprotein and formation of a replication complex associated with intracellular membranes; 4) synthesis of negative-strand RNA using the positive-strand to form a RNA intermediate; 5) production of new positive-strand RNA molecule which can be used for synthesis of new negative-strands, for polyprotein expression or packaging into progeny virions; 6) release of virus from the infected cell (Figure 2).

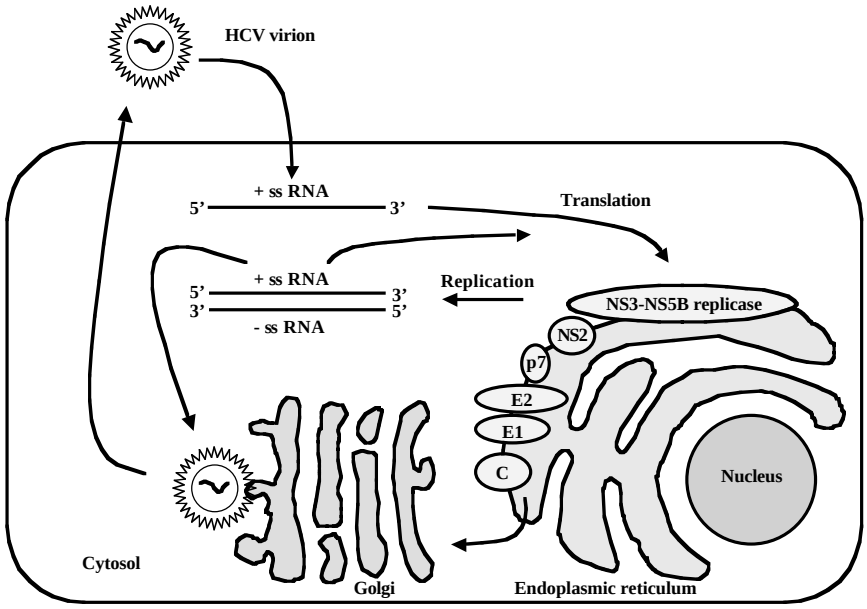

Figure 2. Hypothetical model of the HCV replication cycle.

Virion attachment and penetration

HCV attaches to the cell (i.e. primary hepatocytes) surface presumably through specific receptors. The first protein suggested as a receptor for HCV was CD81, which seems to be a key molecule in the cell surface binding to the E2 glycoprotein [58]. It has also been shown that HCV internalization is facilitated via low-density lipoprotein (LDL) receptors [59] and the virus enters into the cell via endocytosis. Recently, a broadly expressed lipoprotein binding receptor, the human scavenger receptor class B type I was shown to serve as a receptor for HCV [60]. Other reports show that HCV particles bind specifically to L-SIGN and DC-SIGN which functions as capture receptors for HCV and play an important role in pathogenesis and liver tropism [61]. After attachment, the virus enters the cell and is un-coated and the positive-strand RNA genome is released into the cytoplasm. The details of this process in not known.

Polyprotein translation and processing

Once the positive-strand RNA genome is released, it is directly used as a template for protein translation. The translation is mediated by the viral IRES element [54, 62]. Directed

by the IRES, the polyprotein is translated at the rough ER and cleaved co- and post-translationally by host cell signalases and two virally encoded proteases. It has been reported that the HCV proteins most likely form a stable complex associated with intracellular membranes (Figure 3) [63-67].

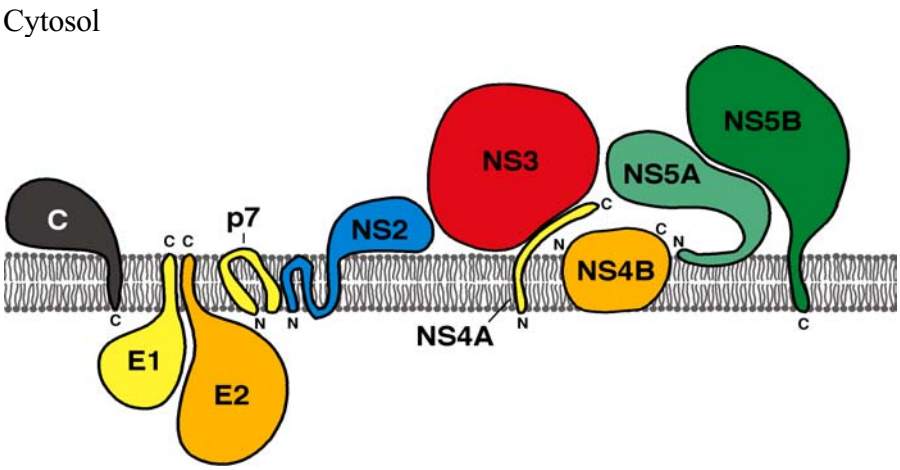

Figure 3. Schematic illustration of the membrane association of HCV proteins. Adapted from Moradpour D et al., Antiviral Research, 2003.

RNA replication

Most or all HCV NS proteins, which are cleaved by the viral proteases, assemble in intracellular membranes, and form the replication complex. The formation of such a complex is a feature typical of positive-stranded RNA viruses like poliovirus or flavivirus [68, 69] and it allows the production of viral proteins and RNA in a distinct compartment.

As previously mentioned, the individual steps during RNA replication are largely unknown. However, by analogy with other members of the Flaviviridae, it is assumed that HCV replication requires the positive-strand RNA to serve as a template for generating the negative-strand RNA. The replication intermediate, which binds to the NS3-5B proteins forms a replication complex, from which the progeny genomic positive-strand RNA is produced. The HCV NS5B RdRp has been shown to be the important factor catalyzing the synthesis of negative- and positive-strand RNA.

Virion assembly and release

Several reports indicate that particle-formation is initiated by the interaction between core proteins with the viral RNA genome. Observations suggest that the viral nucleocapsids acquire their envelope by budding through ER membranes into the lumen of ER. The virus particles are then believed to be exported to the cell surface through the Golgi complex [70] and via the host cell secretory pathway for release outside the cell.

Genetic variability of HCV

Amino acid (aa) similarity among the other members of the *Flaviviridae* family is limited to the serine protease and NTPase domains of NS3 and the RdRp domain of NS5 [71]. These similarities have led to the classification of HCV in a separate genus (*Hepacivirus*) of the family *Flaviviridae*. [72]. HCV appears to be more closely related to pestiviruses than to flaviviruses, based on additional similarity in the nucleotide sequences [52, 55] and secondary structures of their 5' UTRs [73]. Furthermore, both the HCV [54] and pestivirus [74] 5' UTRs appears to serve as IRES for cap-independent translation, unlike flavivirus 5' UTRs, which are thought to bind to ribosomes via typical 5' cap structures [75, 76]. The diversity of HCV has led to further classification into at least six major genotypes (1-6) and numerous subtypes (a, b, etc.) [77, 78] (Figure 4).

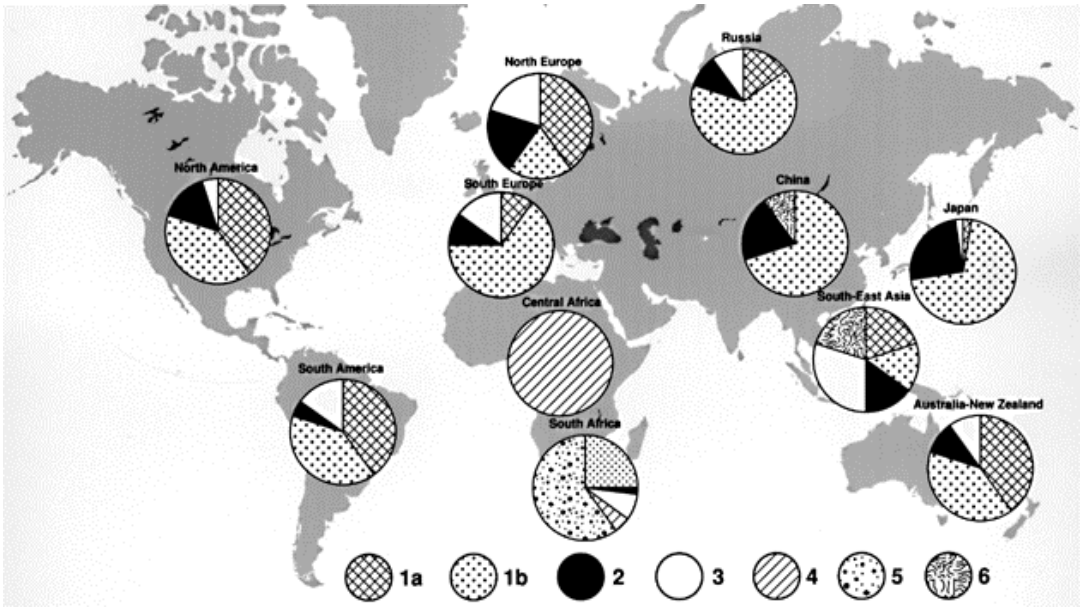

Figure 4. Worldwide distribution of HCV genotypes. Adapted from Forns X and Buhk J, Viral Hepatitis Reviews, 1998.

HCV infection is characterized by a high number of infected patients who develop chronic infections. Although, the mechanism responsible for viral persistence is still largely unknown, the high variability of HCV is widely believed to play an important role. The mutation rate of the virus is high whereby HCV rapidly adapts to the new environment. This is not an unusual feature of RNA viruses, which can be attributed to an error-prone RdRp that lacks proofreading activity. It is estimated that the high rate of viral replication, leads to production of 10^{10} to 10^{13} new virus particles/day and the frequency of spontaneous nucleotide substitutions is high, ~10^{-2} to 10^{-3} substitutions per nucleotide per year [79, 80]. This is regarded as a major obstacle in the development of effective vaccines to prevent or to cure HCV infection [81]. Only a few regions of the HCV genome (i.e. core, NS3, and NS4) display a low genetic variability. These parts may therefore be potential targets for antiviral therapies and vaccine development.

The genetic heterogeneity of HCV is a result of the accumulation of mutations that occur during viral replication and can result in the appearance of quasispecies. Quasispecies are defined as a complex population of closely related, but distinct, virions within a given host. The heterogeneity of HCV in a single host can render traditional preventive approaches ineffective if the immune response does not cross-react between the different viral variants. The biological consequences of quasispecies include the development of escape mutants to humoral and cellular immunity, variable cell tropism (e.g. possibly lymphotropic versus hepatotropic), vaccine failure and rapid development of drug resistance. Another obstacle is that a previous HCV infection may not protect against re-infection [82]. Subsequently, a pre-existing immunity (neutralizing antibodies and cellular immune response) has shown to be of limited importance [83]. The differences between genotypes on nucleotide level is approximately 31-34%, subtypes 20-23%, and quasispecies 1-9% (Table 1) [84].

Terminology	Definition	Nucleotide similarity (%)
Genotype (1-6)	Major genetic group based on similarity of nucleotide sequence	65,7 - 68,9
Subtype (a, b, etc.)	Genetically closely related viruses within nucleotide sequence	76,9 - 80,1
Quasispecies	Complex of genetically variants within individual isolates	90,8 - 99

Table 1. Terminology relating to hepatitis C viral genomic heterogeneity. Adapted from Szabó et al., Pathology Oncology Research, 2003.

Antibodies are essential for neutralization of circulating virus particles, but are of limited efficacy once viruses are inside the host cells. The importance of the antibody response to HCV has been highlighted by the fact that patients with agammaglobulinemia progress rapidly in their HCV infections [85]. Also, HCV strains from such patients do not display the HVR regions within the E2 gene [86]. In contrast, the vigor of the T-cell response to HCV proteins at the early stages of infection has been suggested to represent an important determinant of the outcome of hepatitis C [87, 88]. Vigorous and multispecific CD4-mediated responses directed against structural and nonstructural HCV antigens are present in the acute stage of HCV infection of patients who resolve the infection. In contrast, these responses are significantly weaker or even absent in patients with acute hepatitis C who progress to chronicity. These data suggest that the intensity of the T-cell reactivity at the early stages of infection may be critical to limit the spread of the virus within the infected host and to keep viral replication under control [87, 88]. Similar results have been reported from studies done in chimpanzees, were a strong and vigorous CD4+ and CD8+ T-cell response was important to control and resolve an acute HCV infection [89, 90].

Epidemiology, transmission, and clinical features

Epidemiology

Infection with HCV occurs throughout the world and it is estimated that approximately 170 million people (~3% of the world population) globally are chronically infected with the virus [91, 92]. Of the 170 million people chronically infected, it is estimated that genotype 1 accounts for approximately 50% of these infections. Much of the seroprevalence data are based on blood donors, who represent a selected population. The prevalence of antibodies to HCV in blood donors varies from 0,02% to 1,25% in different developed countries [93]. However, much higher incidence of HCV infection has been found in less developed countries in both the general population and in blood donors [93]. In particular, as many as 4-12% of the general Egyptian population have antibodies to HCV [94]. In Sweden, there are approximately 40.000 individuals who are chronically infected with HCV. The number of new HCV cases annually in Sweden ranges from 3,000 to 3,500 since 1999, according to statistics from The Swedish Center for Disease Control.

Transmission

HCV is primarily transmitted by exposure to infected blood or blood products. Before 1989, HCV was the major causative agent of non-A, non-B hepatitis. The most common ways to spread the virus were through blood transfusions and intravenous drug use. However, since 1990-91 when anti-HCV screening of blood donors became routine, the number of post-transfusion cases of HCV declined to less than 1% among all post-transfusions. Out of the new cases in the late 1990s, intravenous drug use accounted for over 75% of these. Intravenous drug users (IVDUs) not only have the highest prevalence of HCV infection, but also constitute a reservoir of HCV in the community. The incidence of HCV among IVDUs varies between 31% to as high as 98% in different parts of the world [93]. However, in a large proportion of all new cases no recognizable transmission source/route could be identified [95]. Therefore, transmission by yet unknown routes of transmission must occur. Although, sexual transmission does occur, it is rather unusual, with less than 5% of the long-term sexual partners becoming infected [96]. Vertical transmission is also unusual, with a frequency of infection in children of viremic mothers less than 5% [97].

There are several risk factors associated with contracting HCV. The most predominant factors are intravenous drug abuse, haemodialysis, transfusion of blood products, tattooing, high number of sexual contacts, and exposure to organ transplants from HCV positive donors [98, 99].

Clinical features

The incubation period for HCV varies between 14 and 60 days (mean 50 days). The mechanism by which HCV causes human disease is not fully understood. HCV can cause both acute and chronic liver disease. HCV infection is diagnosed by different methods, such as enzyme linked immuno sorbent assays (ELISA) with high sensitivity and specificity for different HCV antibodies, radio-immunoblot assay (RIBA), and by direct detection of viral RNA using the polymerase chain reaction (PCR). However, despite using the techniques available today, it is still not possible to clearly distinguish between an acute and chronic infection.

Most acute infections are asymptomatic, and only 20% of the infections cause jaundice, fatigue, dark urine, abdominal pain, loss of appetite, and nausea. A high proportion

(approximately 60-80%) of acute infections progress to a chronic infection and the prognosis for chronic infections is very variable. Chronic viral hepatitis caused by HCV is defined as persistently abnormal ALT levels for at least six months [100]. Histology examination of liver biopsies from asymptomatic HCV carriers has a range from normal liver histology [101] to chronic active hepatitis and fibrosis [102]. This suggests that HCV is generally not directly cytopathic for the hepatocytes. It is more likely that immune mechanisms are causing much of the liver disease. Steatosis is another frequent histopathological finding in chronic HCV infected individuals [103-106]. Although, it remains unclear whether it is a direct effect related to the viral infection or due to host factors such as obesity and alcohol. Steatosis can be associated with hepatic inflammatory changes and fibrosis. Several studies have demonstrated a relationship between hepatic fibrosis and steatosis during chronic HCV infections [107, 108]. The fat accumulation within hepatocytes of HCV infected individuals, seemed to be one of the earliest predictor of fibrosis [109]. A number of groups have also shown an association between liver steatosis and HCV genotype 3 [110-115], suggesting that specific viral sequences within this genotype may be responsible for the steatosis. *In vivo* [116-120] and *in vitro* [121] studies showed that steatosis was associated with HCV genotype 1b, whereas during human HCV infections show a correlation of steatosis with genotype 3. The reason for these contradictive results is not known. Furthermore, around 20% of patients with chronic hepatitis C develop liver cirrhosis and these patients are at high risk of developing primary liver cancer, hepatocellular carcinoma (HCC) [122]. According to statistics from Center for Disease Control, in USA, HCV is the leading indication for liver transplantation and it is estimated that ≈3% of the infected patients die due to their chronic liver disease. It has been shown that the severity of the disease correlates with abnormal ALT levels and increases with age. Age over 40 years at time of infection, high alcohol consumption, and male sex are factors associated with increased risk of progressive liver disease (Table 2). Other predictive factors are route of transmission and viral genotype [123]. There is some evidence that the infecting genotype may determine the outcome of the liver disease. More severe fibrotic disease has been found in patients infected with genotype 1a and 1b than in those infected with other genotypes [124]. Although, this is somewhat controversial as there are confounding factors, such as duration of infection and mode of transmission, that make the interpretation difficult [125].

Risk factor	Time from infection to cirrhosis (years)
Age >40 years	12
Age ≤40 years	35
Alcohol <50g/day	31
Alcohol >50g/day	24
Male	26
Female	36

Table 2. Factors influencing the progression of HCV infection. Modified from Poynard T et al., Lancet, 1997.

Liver biopsy is the only method to directly assess the degree of inflammation in the liver, the stage of liver disease, and fibrosis. There are two important features in the histological assessment of a hepatitis C virus infection. First, the disease stage (fibrosis) is evaluated, and second, the degree of necro-inflammation and how it changes with time. In order to improve the reproducibility of the assessment, different scoring systems have been developed to quantify viral hepatitis. The two most widely used systems are the Knodell score [126] and a modification of thereof, the Ishak score [127]. These scores combine the two previously mentioned factors for assessment of chronic disease. The differences between the Ishak and Knodell scores are that Ishak expanded the stage score to allow for a better discrimination between degrees of fibrosis.

Treatment

Currently, there is no vaccine available for HCV. Today's treatment, which actually can be curative, is IFN-α as a monotherapy or in combination with the purine nucleoside analogue ribavirin. The outcome of the treatment is depending on several factors, such as the viral genotype, viral load, age, and gender. The most favorable patient to treat is less than 40 years of age, infected by a non-genotype 1 virus, and who has a low viral load (< 10^6 genome copies per mL).

Interferon

Interferons are a family of natural occurring proteins that functions as cytokines in an early response to viral infections. Interferons can display a more or less direct antiviral activity; albeit that it activates cellular and immune functions to induce an antiviral state in exposed cells. Interferons are believed to act by inhibiting the viral protein production by a series of intracellular events, such as increasing RNA degradation and

forcing the cell into apoptosis [128]. Recently, pegylated IFN have become available. Pegylation involves the addition of a polyethylene glycol chain to the IFN protein molecule. There is today two variants of pegylated IFN (pegIFN alfa-2a (40kDa molecule); Pegasys and pegIFN alfa-2b (12kDa molecule); Peg Intron), both have maintained the IFN molecules rapid absorption and rapid time to peak drug level, while also providing a much lengthened half-life. Several studies have shown that pegylated IFNs provide significantly improved response rates compared to standard IFNs [129, 130]. The response to IFN-based treatment is defined by two different parameters, biochemical (serum transaminase levels) and virological (serum HCV RNA). Several studies have also included histological examination of progression of liver disease before and after treatment. There are three potential outcomes after IFN-based treatment of patients with chronic HCV; 1) no response either biochemically or virologically (non-responders; NRs), 2) a reduction of HCV RNA and normalization of ALT during treatment but relapse after cessation of treatment (non-sustained responders; NSRs), or a disappearance of HCV RNA and normalization of ALT that is maintained six months or more after end of treatment (sustained responders; SRs). A sustained virologic response (SVR) to HCV is defined as an absence of HCV RNA by PCR at 24 weeks after therapy. Currently, pegylated IFNs is used as standard treatment for HCV, alone or in combination with ribavirin. One major drawback with IFN-based treatment is the adverse effects which in some cases lead to interruption of treatment. Both standard and pegylated IFN cause similar side effects, such as hematological toxicity, psychiatric disturbances including depression, irritability, insomnia, and suicidal ideation.

Ribavirin

Ribavirin (1-b-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide) is a guanosine analogue that has a broad antiviral activity. The major side effect of ribavirin is hemolytic anemia. Initial studies using ribavirin as a monotherapy for HCV infections revealed a lowering of transaminases levels but it had no, or a very limited effect on the viral load [131]. The exact mechanism of action of ribavirin in the therapy of chronic HCV infections is not known. However, several potential mechanisms of action have been proposed. Ribavirin is believed to act against HCV by enhancing T-cell mediated immune responses to HCV by shifting the balance towards a T-helper 1 response [132, 133] inhibiting cellular inosine monophosphate dehydrogenase, thereby decreasing the

intracellular guanosine triphosphate pool needed for viral RNA replication [134]. Ribavirin may also directly inhibit the HCV RdRp [135] and acting as a RNA virus mutagen, thereby reducing viral fitness [136]. As detailed below, ribavirin is almost exclusively used in combination with interferons.

Combination therapy with interferons and ribavirin

The use of IFN and ribavirin in combination therapy was shown to be more effective than the use of IFN alone for treatment of HCV [137]. When using the combination therapy, approximately 40% the treated patient achieved a SVR, compared to only 20% when treated with IFN alone. Studies combining pegIFN alfa-2b with ribavirin showed a 54% SVR compared to 47% SVR with standard IFN plus ribavirin [138]. Patient infected with HCV genotype 1, did respond poorer to therapy compared to non-genotype 1 infected. Although, these patients had an improved SVR of 42% compared to 33% with standard IFN. Combination therapy using pegIFN alfa-2a plus ribavirin has also showed improved SVR in patients treated [139]. Results from different studies using both pegIFN alfa-2a and alfa-2b show remarkably similar effectiveness in SVR rates, albeit their different structure. Today, by using the pegylated IFNs in combination with ribavirin, the cure rates is around 75% or more in genotype 2 or 3 infections, and 40% in genotype 1.

However, nonresponders and relapsers after cessation of therapy are frequent, and only a proportion (≈40% genotype 1, ≈75% non-genotype 1) of treated patients have a SVR with long-lasting normalization of liver functions. Therefore, more effective therapy regimes are needed, and the development of new antiviral compounds and vaccines is of crucial importance to decrease the spread and cure already infected individuals.

Therapies in development for HCV

Several new drugs are under development to target key components of the viral life cycle. Attempts are made to develop inhibitors of the HCV serine protease, helicase, and RdRp. Recent studies have reported different serine protease inhibitors that have been evaluated both *in vitro* and *in vivo*. One of these inhibitors, were both effective and rapid in reduction of HCV RNA levels in plasma during therapy [140]. However, after cessation of therapy, the HCV RNA levels rapidly increased to levels equal as before start of treatment. *In vitro* studies have shown that both evaluated protease inhibitors

developed drug-resistant mutations [141]. These new drugs will probably meet the same problems of viral resistance that we today see in the anti-viral treatment of HIV and HBV infected individuals.

A new strategy to treat chronic HCV infections may be the use of RNA interference (RNAi). RNAi is a cellular process of sequence-specific, post-transcriptional silencing of genes in plants, insects, and in animals [142-145]. Small interfering RNA (siRNA) around 21-23 nucleotides long can initiate the degradation of target RNA complementary to the siRNA. The degradation is mediated through cleavage by cellular ribonucleases [143]. RNAi has quickly become a useful method for analysis and regulation of gene functions, and the technique has potential to be used in therapeutic applications. By introducing siRNA into mammalian cells, the siRNA can specifically silence cellular mRNA without induction of non-specific IFN responses that are activated by longer RNA duplexes. It was recently shown that siRNA could suppress both HIV and poliovirus replication [146, 147]. In the HCV replicon model system, siRNA was shown both to suppress protein synthesis and block viral replication [148-150]. Thus, the use of siRNA may provide an additional therapeutic option to use alone or in combination with already existing antiviral therapy.

Another new approach under development for treatment of HCV is therapeutic vaccination and genetic vaccination using virus vectors expressing parts of the viral genome. These new approaches will be discussed in a later part of the text.

Immune response to HCV proteins

HCV enters a host either directly through transfusion of contaminated blood products or less efficient by perinatal or sexual transmission. The virus reaches the liver via the hepatic artery or the portal vein and enters the hepatocytes, its preferred site of replication. Despite that most HCV infections elicit specific immune responses clearance of the virus occurs in only a minority of infected subjects. Low levels of viral antigens, together with high viral turnover and high mutation rates, leads to rapidly changing antigenic epitopes. This may hamper, or even prevent, the development of effective antiviral responses [151]. However, circulating HCV-specific T-cells have been demonstrated as early as 3-4 weeks after infection, whereas HCV-specific antibody responses occur much later, between 7 and 31 weeks after infection [152]. Studies in chimpanzees suggest that the spread of HCV in the acute phase is so rapid that it can outpace the specific immune response [90]. Also, depletion of CD4+ T cells prior to HCV

challenge causes chronicity highlighting the importance of the CD4+ T cell response [89]. Although, the antiviral immune response is limited in efficiency, it plays an important role in pathogenesis by driving tissue damage. HCV is, as far as we know today, mainly a non-cytolytic virus and in acute infections, the liver damage is associated with the immune response, but independently of viral replication [153, 154]. When viral infection occurs, the innate immunity non-specifically combats pathogens. Natural killer (NK) cells and NK T-cells (NKT) activate and kill cells that undergo changes caused by the infection. Other cells involved in the innate defense systems are granulocytes, macrophages and dendritic cells (DCs). One of the earliest and most common viral products in infected cells is double stranded RNA, and most cells respond with synthesis of type I IFNs. Type I IFNs (i.e. IFNα/β) is produced by the infected cell, which leads to a suppression of viral replication and increased apoptosis. If the viral infection is not controlled by these early defense mechanisms, an adapted immunity is required and activated to specifically kill the virus through different mechanisms, such as neutralizing antibodies and T-helper (Th; CD4+) and cytotoxic T- (CTL; CD8+) cells (Figure 5). These specifically activated responses are key components in eliminating the virus. Neutralizing antibodies bind to viral particles in body fluids and eliminate them (humoral immunity), whereas CTLs and NK/NKT cells eliminate cells infected with viruses. Effective cellular immune responses are dependent on direct interactions between T-cells and cells bearing the antigen that the T-cell recognizes. The CTL release a protein called perforin, which puches holes in the cellular membrane of the target cell, and another protein called granzyme, activates death substrates in the target cell. Perforin and granzyme are believed to cooperate during the killing of the virus-infected cells [155-158]. Activated CTLs also show increased expression of Fas ligand (FasL) and tumor necrosis factor (TNF)-α. If their target cells are sensitive to FasL or TNF-α, activated CTLs damage these cells. This is mediated through the release of apoptotic signals through FasL-Fas antigen and TNF-α systems [159, 160]. In addition, virus-infected cells may also be killed by NK- or NKT-cells. The killing is mediated by release of perforin and granzymes. NK/NKT-cells are effectively activated by stimulation by type I IFN and IL-12. Perforin and granzyme mainly damage cells infected by viruses, whereas FasL and TNF-α attack cells with an acquired sensitivity as a result of cellular damage. [161]. Although neutralizing antibodies and CTLs are direct involved in killing of virus-infected cells the production of antibodies, activation and proliferation of CTLs are controlled by the T-helper cells. They recognize viral

antigens that are presented by antigen-presenting cells (APC), such as DC, macrophages, and B-cells. The T-helper (Th) cells are divided into two functional classes; Th-1 and Th-2 cells, according to the cytokine profiles. When activated, Th-1 cells produce interleukin (IL)-2, IFN-γ and tumor necrosis factor (TNF)-α which accelerate the activation and proliferation of CTLs and NK cells [162, 163]. Th-2 cells produce IL-4, IL-5, IL-6, and IL-10, which promotes B-cells to differentiate into antibody producing plasma cells [164]. APC produce IL-12, which acts on Th1 cells, CTLs, and NK cells, leading to viral elimination and suppression of viral replication.

Epitopes of viral proteins are presented by the major histocompatibility complex (MHC) class I molecules on the surface of infected hepatocytes. Extracellular viral components are taken up by macrophages, Kupfer cells, or dendritic cells. These antigens are then presented to T-cells via major histocompatibility complex class II in lymph nodes or possibly within lymphoid-like structures that develop in the liver in response to local cytokines [153, 154]. Priming by CTLs by cross-priming using viral components from infected hepatocytes that have undergone apoptosis and which have been processed by dendritic cells and presented to T-cells may be of importance in the effectivity of the priming event [165].

During the acute phase of a HCV infection, it has been shown that strong CD4+ and CD8+ T-cell responses to HCV antigens are detected in patients who clear the infection as compared to those who develop chronic infection [166, 167]. When analyzing the proliferative CD4+ T-cell response in patients with self-limited infection, the immune response shows much more vigor compared to the response in chronically infected individuals [87, 88, 168, 169]. The CD4+ T-cell response is directed against several different HCV proteins, such as core, E2, NS3, NS4, and NS5 [170]. It has been reported that patients with self-limited HCV infection has a strong and sustained CD4+ T-cell response to NS3 (Figure 6) [87, 88]. Patients with self-limited acute hepatitis C displayed a Th1 cytokine profile upon HCV antigen stimulation, while a Th2 cytokine profile was observed in patients who developed a chronic infection [168, 171]. Therefore, the induction and maintenance of an HCV-specific CD4+ T-cell response may be an important factor in the defense against HCV infections.

Strong and vigorous CD8+ T-cell responses have also been associated with viral clearance in acute hepatitis C. Viral clearance correlated with the presence of a strong and multispecific CTL response against multiple HCV proteins in both chimpanzees [172] and humans [173, 174]. The frequency of CTLs specific for a given HCV epitope has been shown to be in the range of 0,01%-1,2% of the total pool of peripheral blood CD8+ T-

cells during chronic infection [175] and 2%-8% during the acute phase of HCV infection [174]. However, to obtain a complete view of the total number of HCV-specific T-cells, one has to analyze all potential epitopes in the context of all MHC molecules of a given host. Nevertheless, these results may still give an indication of the situation in an infected individual. It has been suggested that patients with chronic HCV infection undergoing IFN-alpha therapy alone or in combination with ribavirin may clear the infection more often if they had a strong NS3-specific Th1 T-cell response, as compared to those with a weak Th2 type of T-cell response to NS3 (Figure 6) [176, 177]. Most studies have found that the CD4+ and CD8+ T cell response to the NS3 protein correlate with clearance and control of the infection [87, 88, 177]. Thus, T-cells directed against the NS3 domain seem to be of particular importance both during the acute and chronic phase, as well as during antiviral therapy. NS3 should therefore be considered as a possible target in vaccine development.

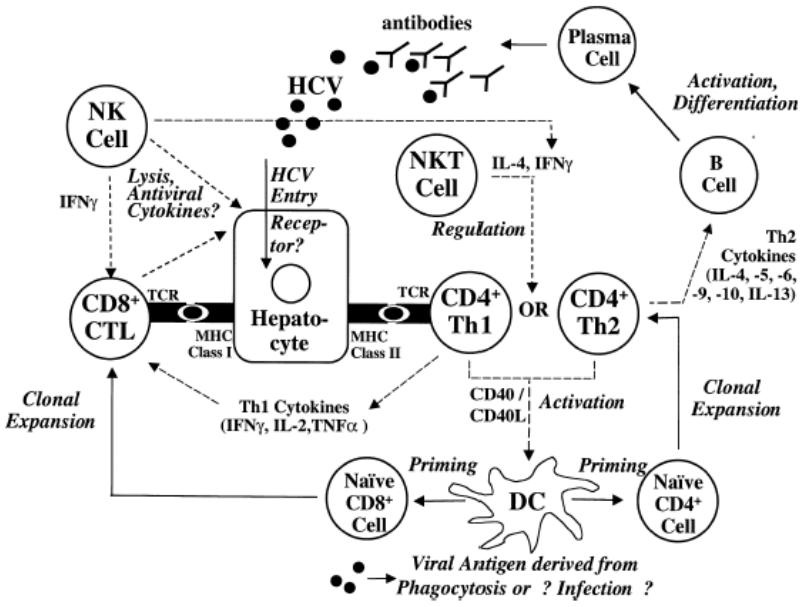

Figure 5. Components of the HCV specific immune response. Adapted from Mizukoshi E and Rehermann B, 2001, J Gastroenterology.

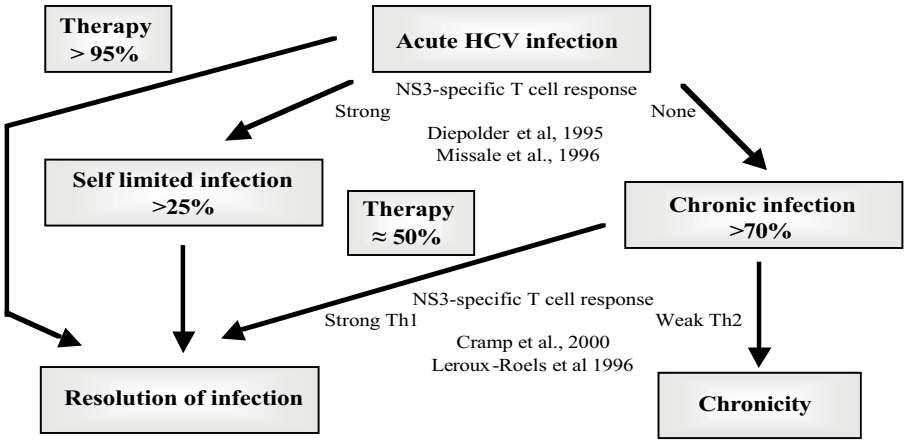

Figure 6. Schematic illustration of HCV pathogenesis.

GENETIC VACCINATION

Genetic vaccination is a rather new approach where manipulated genetic material is used to generate an immune response targeted against a desired antigen. The genetic vaccine may be delivered as naked DNA, by recombinant viruses or bacteria producing the antigen of choice or by self-replicating viral vectors [178, 179].

DNA vaccines

DNA vaccination has become a rapidly growing field in vaccine technology following reports at the beginning of the 1990's that injection of plasmid DNA with the gene of interest expressed under the control of an eukaryotic promoter induces an immune response to the plasmid-encoded antigen [180-184]. In contrast to vaccines that employ recombinant viruses, plasmid vaccines consist only of DNA or RNA, which are passively taken up by cells and translated into protein. The expression of the encoded antigens by the host cell is one of the major advantages of this approach because it is believed that it mimics natural infection. Similar to live or attenuated viruses, DNA vaccines effectively engage both MHC class I and MHC class II pathways allowing for the induction of both $CD8^+$ and $CD4^+$ T-cells (Figure 7) [185].

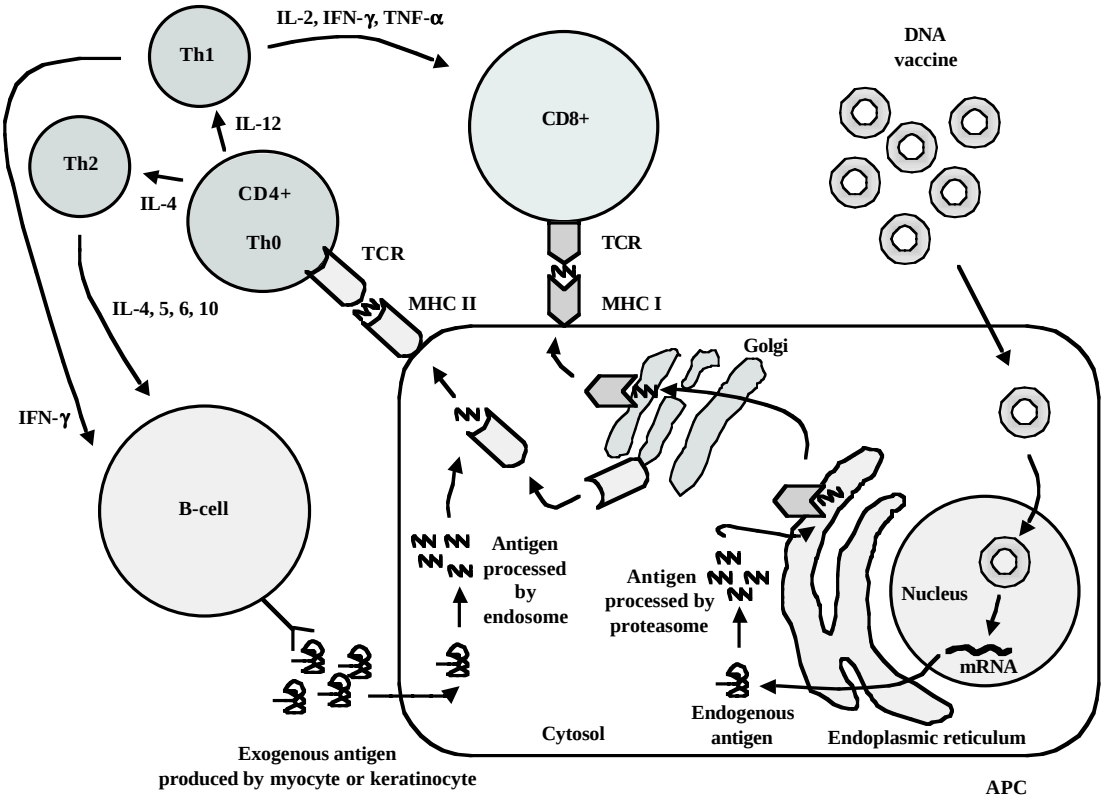

Figure 7. Schematic illustration of immune cell activation by DNA vaccines.

In contrast, antigens present in soluble form, such as recombinant proteins, mainly induce antibody and CD4+ T cell responses. DNA vaccines can circumvent many of the problems associated with recombinant protein-based vaccines, such as high costs of production, difficulties in purification, incorrect folding of antigen and poor induction of $CD8^+$ T-cells. DNA also has clear advantages over recombinant viruses or self-replicating viral vectors, which are plagued with the problems of pre-existing immunity, risk of insertion-mutagenesis, loss of attenuation or spread of inadvertent infection [186, 187]. However, DNA vaccination has in many cases been hampered by poor efficacy and immunogenicity. Various strategies are being developed to improve immune responses induced by genetic vaccines and in particular DNA vaccines. Genetic vaccines can be delivered into the host by several routes and methods (mucosal and pressure injectors, gene gun, electroporation etc). Needle-injection into muscle tissue and into the skin is the most commonly used method [184, 188, 189]. Since myocytes are able to passively take up some of the plasmid injected into the muscle the mechanism of intramuscular immunization seemed very straightforward. There are some reports that indicate that this uptake was enhanced when muscle fibrils were

recovering from tissue damage induced by the injection of cardiotoxin [190, 191]. However, the use of cardiotoxin is for obvious reasons not possible to use in humans. On the other hand, muscle is not considered an immunologically relevant tissue as myocytes lack the characteristics of professional APC such as MHC class II expression, co-stimulatory molecules or marked cytokine production. However, they may serve as efficient factories for the production of antigens and function as suppliers of antigens to APCs [192, 193]. The priming of T-cells occurs after that APCs have processed the viral antigen, or the antigen expressing cell, and presented it on MHC class I and II molecules to CD4+ and CD8+ T-cells, a process called cross-priming [192, 194-196]. Other methods to deliver DNA vaccines are by the use different delivery devices, such as gene gun, where the DNA vaccine is coated onto gold particles that are injected transdermally by particle bombardment. By using gene gun instead of needle injection it was shown to be possible to lower the dose of DNA to a few nanogram, and still generate similar results as with 50-100μg given intramuscularly [197, 198]. One advantage by injection into the skin is that the skin contains a high number of DCs (i.e. Langerhans cells), which are good APCs. The DNA vaccine can also be given as a viral or bacterial vector producing the desired antigen. Langerhans cell may pick up an antigen and migrate to local lymphoid tissues to initiate an immune response [199, 200]. When DNA is given to mice by the use of gene gun, approximately 20% of the cells in the target area are transfected [201]. The penetration of gold particles coated with DNA results in tissue-stress, which may contribute to recruiting and activation of DCs. The number of DCs in the local lymphoid tissues increases after gene gun immunization. Since, most of the DCs do not contain the DNA plasmid, the gene gun bombardment of plasmid free gold particles per se may therefore have an adjuvant effect [202].

There are several ways to improve the immunogenicity of the vaccine by plasmid alterations (gene expression, co-expression of immune modulators, such as chemokines, cytokines, immunostimulatory sequences etc), enhancement of plasmid stability (encapsulations, formulations etc), or slow release formulations. Since plasmid DNA are produced in bacteria they contain sequence motifs termed *CpG* which are recognized as foreign by the immune system and thereby induces a non-specific cytokine production, which may enhance the overall immune response targeted against the plasmid encoded antigen [203-207]. It has also been reported that priming with DNA and boosting with a recombinant virus or protein encoding the same antigen (prime-boost regimen), may generate a more potent immune response against the desired antigen compared to using DNA alone [208-211]. It is worth noting that different delivery

routes of the vaccine may prime different types of immune responses [212-214]. For example, intramuscular injection is associated with a Th1 response [207, 215-217], whereas transdermal injection using gene gun primes a more Th2 type of response [217, 218]. However, the Th response may also be dependent on the antigen and dose. Therefore, it is important to evaluate which route of delivery and dose of antigen that works best for each individual antigen. The amount of DNA used should also be carefully choosed, since it may affect the type of immune response induced. Low amount of DNA given intramuscularly has been reported to induce an immune response characterized by a Th2-like phenotype [219], whereas a high dose induces predominantly a Th1-like immune response.

Clinical trials for a number of diseases (HIV, malaria, influenza, HBV) have demonstrated that DNA vaccines are safe [220-222], generate both humoral [220, 221, 223-225] and cellular [223-227] immune responses. Although, results from these studies have shown that these vaccines still have a limited potency.

Recombinant viruses and bacteria as vehicles for delivery of genetic vaccines

The genetic vaccine can also be given as a viral or bacterial vector producing the desired antigen. Recombinant viruses have been demonstrated to be a powerful system to use to deliver vaccine genes. Several different viral genes have been delivered using recombinant Retroviruses [228-230], Adenoviruses [231, 232], Adeno-associated viruses [233], Lentiviruses [234, 235], Herpesviruses [236], Poxviruses [237-239], and Alphaviruses [240].

Bacteria as a delivery system for genetic vaccines are another approach. One advantage is that bacterial vectors can be used for oral administration to induce a mucosal immunity. Several different attenuated strains of bacteria have been evaluated for use as delivery models, such as *Shigella* [241-243] and *Salmonella* [244-246]. There are major safety concerns due to bacterial virulence and a pre-existing immunity.

Viral vectors with the property to self-replicate are a favorable approach to use as candidate vehicles in development of genetic vaccines. Self-replicating genetic vaccines were established to overcome the poor efficacy that is the major problem with DNA- based vaccines. Different self-replicating genetic vaccines have been generated from the Alphavirus genus of the Togaviridae family, which includes the Sindbis virus, Semliki forest virus (SFV), and Venezuelan equine encephalitis (VEE) virus. The system based on the SFV replicon has been widely used as a model to study viral

processes and for expression of foreign genes [240, 247]. Also the effective use of Sindbis- and VEE-based vectors has been reported [248-253].

SFV causes a lytic viral infection, which kills the infected cell. In the SFV replicon the single-stranded positive RNA genome is encapsidated by a protein-lipid envelope and its RNA genome can serve as an mRNA for direct translation. The viral RNA encodes its own RNA replicase, and the viral polyprotein is autoproteolytic processed into four different nonstructural proteins (nsP1-nsP4) [254, 255]. The nonstructural proteins are responsible for the viral replication. Upon infection, the virus first translates the replicase complex, which initiates replication of the viral RNA by generating a negative-strand. The negative-stand is then used as a template for a new positive RNA strand encoding the structural proteins. The SFV vector system offers the possibility to replace the structural proteins with the antigen of choice, allowing for a self-replicating genetic vaccine. A schematic illustration of SFV self-replicative genetic vaccine life cycle and immune activation is outlined in figure 8. When the structural proteins are replaced with a gene of interest, the SFV replicon becomes replication deficient and can only infect once and produce viral encoded antigens until the cells dies. The replication and transcription of the viral RNA is effectively driven by the SFV replicase complex, which enroll almost all ribosomes in the infected cell to synthesize the viral encoded proteins. The viral encoded antigen product can constitute of up to one fourth of the total cell proteins [240]. SFV has a broad host range and several studies have shown that it is possible to infect mammalian, avian, reptilian, amphibian, and insects cells [256, 257]. Concerns about the safety by using self-replicating genetic vaccines should be addressed. The viral vector itself is often antigenic. In fact, the antigenic property of the vector may in some instances benefit the desired immune response, but problem with pre-existing immunity due to previous infection or vaccination may limit the use of the vaccine. SFV-based genetic vaccines have been shown to be more, or equally, effective in inducing both humoral and cellular immune responses compared to most non-replicating DNA- and RNA-vaccines [258-260]. A main difference between self-replicative genetic vaccines and DNA- or RNA-vaccines is that the virus-like RNA-replication inside the infected cells may trigger a series of danger signals, which can adjuvant the immune response [261]. The self-replicating vaccines may also induce apoptosis of the infected host cell [259, 262], which in turn activate the immune system through DCs [263]. During SFV replication, dsRNA intermediates are generated which activates the IFN response of the cell. Virus-derived dsRNA is known to trigger and activate a number of

molecules (for example heat shock proteins (HSPs)) leading to a potent activation of humoral and cellular immune responses [264, 265] (Figure 8).

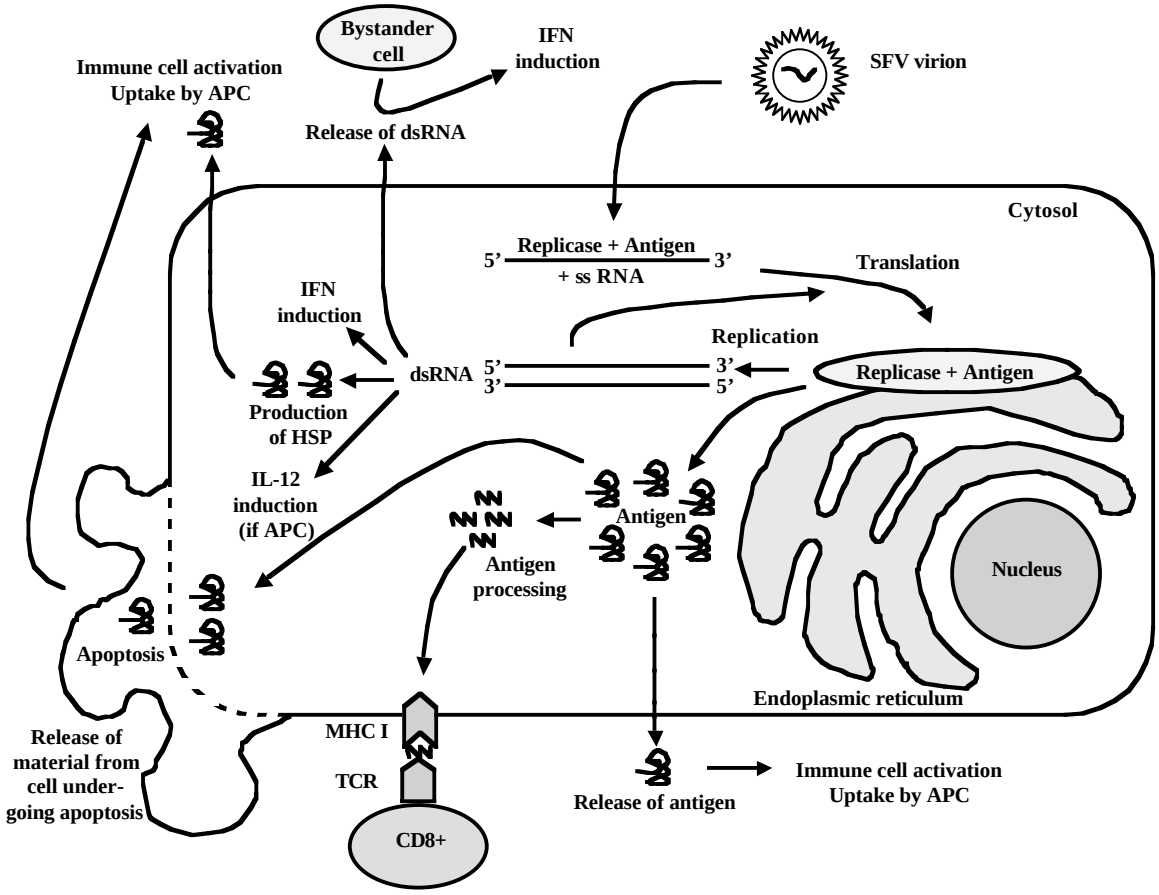

Figure 8. Schematic illustration of the SFV self-replicative genetic vaccine life cycle and immune activation.

There are several advantages in using the SFV replicon: it allow high level expression of the desired antigen, a broad host range, and a cytoplasmic replication of the antigen mRNA which is independent of the host replication system.

HCV-specific immune responses primed by genetic immunization using DNA-vaccines or recombinant viral vectors

Recent reports show that DNA-immunization in mice using both the structural and/or nonstructural HCV proteins can generate humoral and cellular immune responses [266-274]. The use of DNA-plasmids encoding the nonstructural proteins [268, 270-272] seems to

generate more potent humoral and cellular immune responses compared to responses induced by the structural proteins [266, 267, 275]. A major problem with genetic immunization against HCV has been the rather limited immunogenicity. Attempts to enhance the potency of genetic vaccines have been made by various prime-boost regimens [276], modification of gene-expression and co-expression using immune modulators [271, 277, 278], electroporation [279, 280], and the use of viral vectors [232, 270, 271, 281].

Since the chimpanzees are the only infectious and reliable experimental animals to study HCV pathogenesis, vaccination studies in such models may contribute with important information. A recent report by Forns *et al* showed that E2 DNA-immunization in chimpanzees protected against homologues challenge with HCV [282]. Another report by Choo *et al* [283] demonstrated that prophylactic vaccination using E1 and E2 proteins generated potent humoral immune responses that protected against homologous challenge. However, a homologous challenge will only generate limited information due to the high genetic diversity of HCV. Therefore, a more *in vivo* like situation would be to challenge with a heterologous strains of HCV. In a study by Rollier *et al* [284] they showed that vaccination with DNA and boosting with protein encoding all structural plus NS3 proteins of HCV could prime specific humoral and cellular immune responses. Importantly, the vaccines possibly primed a Th1-like immune response against NS3 and E1, which previously has been shown to be of crucial importance in clearance of an acute infection or ongoing chronic infection during antiviral treatment in humans and chimpanzees [87, 88, 176, 177, 285]. These findings support the concept of the NS3 domain of HCV as a potential vaccine target.

The field of DNA- and viral vectors as vaccines is still growing, and new vaccines have demonstrated promising results and a number of clinical trials are ongoing. Overall, the DNA vaccines and self-replicative viral vectors still have a major problem, the limited immunogenicity in humans. This problem has to be solved in the future to enable the use of genetic vaccines in humans.

MODEL SYSTEMS TO STUDY HCV

Cell culture models

Primary cell cultures and cell lines

Several attempts have since the discovery of HCV in 1989 been made to establish a reliable cell culture propagation system. A number of experimental systems have been

described were attempts to infect primary cell cultures or cell lines have been made or the cultivation of primary cells from chronically infected patients. However, thus far neither of these attempts has shown reproducibility nor robust RNA replication. Several groups [286-289] have described infection of primary hepatocytes from both humans and chimpanzees. Although the results have been highly controversial [290, 291].

Replicon

A breakthrough was made when it for the first time was described that a group had developed a subgenomic self-replicating HCV RNA system (replicon) [25]. The replicon was derived from a HCV consensus genome from a chronic infected patient. By deletion of the structural regions core to p7 or NS2, and insertion of a gene encoding the selection marker neomycin phosphotransferase (neo) and the IRES from encephalomyocarditis virus (EMCV). The generated replicons were bicistronic with translation of the first cistron (neo) being directed by the HCV IRES and translation of the second cistron (NS3-5B) by the EMCV IRES. By transfecting the human hepatoma cell line Huh-7 with these replicons and using selection with neomycin sulfate (G418), a number of colonies were obtained. The replication of the replicons allowed for detection using Northern blot. Both positive and negative strands were detected in these cell lines. Detection of viral protein were investigated and found exclusively in the cytoplasm associated with the ER membrane, as suggested in previous studies [292]. A recent report identified the formation of a membrane-associated replication complex, composed of the HCV nonstructural proteins, replicating viral RNA, and altered cellular membranes, designated the membranous web. The membranous web is a feature of many positive-strand RNA viruses, which functions as a replication complex [293-298]. The membranous web has often found to be closely associated with the rough ER [292]. It is speculated that the membranous web is derived from membranes of ER.

A long-term follow up on these replicons-harboring cell lines did not show any cytopathogenicity [299]. Further studies showed that the replicons, which had a high level of replication, had adapted mutations [300-302] and these mutations were found throughout the polyprotein [302]. Some mutations enhanced the viral replication by 500 and up to 20.000 fold [300]. However, the reason for these cell culture adaptive mutations is still unknown, but they may develop due to needed modulations of the NS3 NTPase/helicase and NS5B RdRp activities for optimal enzyme functions [302]. When these mutations have been introduced to infectious clones that are used to infect

chimpanzees these effects are quite the opposite, in that the clone is unable to generate a productive infection [303]. The use of cell culture adapted replicons have enabled studies of new antiviral compounds directed against targets such as NS2/3 and NS3/4A proteases, the NS3 NTPase/helicase, and the NS5B RdRp. These replicons are now serving as a model system for development and evaluation of antiviral drugs. For example, NS3-specific protease inhibitors have been evaluated both in cell culture adapted replicons and in clinical trials with promising results [140, 141]. It is worth noting that the replicons can develop drug-resistance mutations, which suggest a potential problem when using them as antiviral therapy in humans.

One should be aware of several factors when evaluating new drugs using these replicons. First, HCV RNA replication is tightly coupled to host cell proliferation [299] and, thus, compounds inhibiting host cell growth will also reduce the level of replicon RNA. Second, many replicons lack the structural proteins of HCV which themselves may influence the antiviral activity of a compound or contribute to the development of antiviral resistance. Third, as previously mentioned cell culture adaptive mutations may have different effects in transfected cells as compared to infected chimpanzees or humans. Taken together, replicons enable a unique opportunity to study new antiviral drugs *in vitro*.

Animal models

The chimpanzee model

Currently, the only reliable experimental animal model that reproducible can be infected with HCV is the chimpanzee. Due to the rarity, high maintenance costs, and ethical reasons, the used of this model is limited. In most chimpanzee studies, a limited number of animals have been used, which makes the results hard to interpret. Therefore, several attempts have been made to establish an infectious small animal model. Later in the text, existing small animal models will be discussed.

The chimpanzee model has generated valuable insights into HCV, since it is possible to follow the animals from the time of infection and measure different parameters during the acute and chronic phases of the infection. Approximately 60% of the infected chimpanzees clear the infection [304], which is a very high clearance rate compared to humans. This is the first signal that HCV infection in humans and chimpanzees may differ. The second signal is that chimpanzee derived HCV strains do not display a high

genetic variability within the HVR-1 and HVR-2 regions of the E2 protein [305]. Contradictory to the well know observations from human HCV strains. Thus, data from the chimpanzee model on immune mechanisms leading to clearance or persistence should be interpreted with care.

The high rate of viral clearance in chimpanzees makes it a useful animal model for probing the mechanism of clearance. Results from a chimpanzee study showed that a strong T-helper response was important for resolution of infection, and especially Th-cells specific for one epitope within the NS3 domain [285]. The importance of a pre-existing memory response was analyzed in chimpanzees and the results suggested a potential role in controlling viremia [89]. Another report showed that CD4+ T cells are essential for protection against HCV re-infection. Depletion of CD4+ cells in chimpanzees resulted in persistence and low-level viremia, despite an intra-hepatic memory CD8+ T-cell response. CD8+ T-cells were unable to control HCV replication in absence of CD4+ T-cell help, which resulted in viral escape mutations in MHC class I epitopes and failure to resolve infection [89]. Chimpanzees challenged two times with HCV and who cleared both infections, had T-cells targeted against all HCV proteins [89]. In addition, neither of these animals had any detectable envelope protein specific antibodies. These results were similar to those reported in other acute resolving HCV infections of chimpanzees [90, 172, 306] and humans [174, 307], with resolution correlating with strong and sustained T-cell response. Data from the chimpanzee model clearly suggest that previous infection does not protect against re-infection, although it seems that a pre-existing memory immune response and neutralizing antibodies are of importance to solve infection. In human HCV infection some data suggest that a previous infection may partially protect against re-infection or at least development of chronicity [83].

Mouse models

Several transgenic mouse models have been established to study the characteristics of the different HCV proteins. Mouse models have also been established were the mouse livers have been repopulated with human hepatocytes, which enables studies of human hepatotropic viruses, such as HCV, within the mouse.

Transgenic mouse models

Several transgenic mouse lineages have been established to enable studies of the *in vivo* effects of different HCV proteins. Today's existing HCV transgenic models are: 1) full-length HCV genome [116-118, 308], 2) core [119, 120, 309, 310], 3) core-E1-E2 [311, 312], 4) core-E1-E2-p7 [118], 5) E1 and E2 [313], 6) E2 [309], and 7) NS5A [314, 315]. The current existing HCV transgenic mouse lineages and their characteristics are summarized in table 3.

Transgenic mouse lineage	Mouse strain	HCV genotype (gt)	Promoter	Inflammation (+/-) / onset age (months)	Steatosis (+/-) / onset age (months)	HCC (+/-) / onset age (months)	RNA detection	Protein detection	Immuno histochemistry / intra-hepatic localization	References
Core→NS5B	C57BL/6	gt 1b	hSAP	- / -	- / -	- / -	Core-NS5B	Core	Core / cytoplasmic	Matsuda J et al., 1998
Core→NS5B	C57BL/6	gt 1b	Alb	-/ -	+ / 10	+ / 13	Core-NS5B	-	- / -	Lemon SM et al., 2000 Lerat H et al., 2002
Core→NS5B	C57BL/6	gt 1b	α1-anti-trypsin	+ / NP	+ / NP	NP	Core-NS5B	Core	Core / nucleus and cytoplasmic	Blindenbacher A et al., 2003
Core-E1-E2-p7	C57BL/6	gt 1b	Alb	-/ -	+ / 9-15	+ / 13	Core-E1-E2-p7	Core E2	Core / cytoplasmic E2 / cytoplasmic	Lerat H et al., 2002
Core-E1-E2	FVB	gt 1b	MUP / Alb	-/ -	- / -	- / -	Core-E1-E2	Core	Core / cytoplasmic E2 / plasma membrane and cytoplasmic	Kawamura T et al., 1997
Core-E1-E2	C57BL/6	gt 1b	H2-K	+ / 10	- / -	- / -	Core-E1-E2	-	Core / cytoplasmic	Honda A et al., 1999
Core	C57BL/6	gt 1b	HBV reg. element	- / -	+ / 3	+ / 16	NA	Core	Core / nucleus and cytoplasmic	Moriya K et al., 1997, 1998
Core	C57BL/6 x SJL	gt 1a	MUP	- / -	- / -	- / -	Core	Core	- / -	Pasquinelli C et al., 1997
Core	C57BL/6	gt 1b	hSAP	- / -	- / -	- / -	NA	Core	- / -	Kato T et al., 2003
E1-E2	CD1	gt 1b	HBV reg. element	- / -	- / -	- / -	E1-E2	E1 E2	E1 / cytoplasmic E2 / cytoplasmic	Koike K et al., 1995
E2	C57BL/6	gt 1a	Alb	- / -	- / -	- / -	E2	E2	- / -	Pasquinelli C et al., 1997
NS5A	FVB	gt 1a	MUP / ApoE	- / -	- / -	- / -	NS5A	NS5A	NS5A / cytoplasmic	Majumder M et al., 2002, 2003

NA = not analysed
NP = data not published
α1-antitrypsin = Alpha1-antitrypsin
Alb = Albumin
ApoE = apolipoprotein E
HBV reg. element = HBV regulatory element
hSAP = human serum amyloid P component
MUP = mouse major urinary protein

Table 3. Existing HCV transgenic mouse lineages and their characteristics.

One of the most well characterized transgenic mice expressing HCV proteins is the core transgenic lineage. Studies in these mice show no immediate toxicity from the expression of the core protein. However, the most significant phenotypic changes observed in most core transgenic mice were the induction of steatosis and HCC, both which are characteristics of chronic HCV infections [116-120]. However it should be noted that not all core transgenic mice develop steatosis and HCC [309-312]. The reasons underlying these differences are unknown, but it can possibly be attributed to insufficient levels of protein expression or the specific genotype of HCV used (gt 1a vs

gt 1b). It should be noted that Kawamura *et al* [311] and Pasquinelli *et al* [309] did use mice with different genetic background as compared to the mice used by Moriya *et al* [119, 120] and Lerat *et al* [118]. Thus, the genetic background may be a factor determining disease development in HCV transgenic mice. It has also been postulated that the core protein may have some effects on the host metabolism. Increased lipid peroxidation and mitochondrial DNA damage, both indicators of increased reactive oxygen species (ROS), have been reported in some core transgenic mice [316]. In this report, the authors suggest that development of HCC in core transgenic mice and HCV infected patients is due to generation of ROS in the liver and subsequently DNA damage. It was recently shown *in vitro* that core protein induces oxidative stress through interaction with the mitochondria. The core protein has also been reported to interact and modulate the host immune system [317-319] and to act as transcriptional regulator and affect the proliferative ability of the cells [19-24]. Expression of core protein in the liver of transgenic mice, at a level similar to that in human chronic HCV patients [320] was shown to be correlated with an impaired ability of insulin to lower the plasma glucose level. This results in development of insulin resistance, which finally leads to type 2 diabetes [321]. In fact, it has been reported that a linkage between type 2 diabetes and chronic HCV infections may exist [322, 323]. Observations in the core transgenic mice may suggest that HCV infection confers insulin resistance and additional factors such as obesity, cirrhosis, aging [324], gender [325], or possible inflammation may contribute to the development of overt diabetes. Shintani *et al* [321] showed that insulin resistance preceded the occurrence of hepatic steatosis, indicating that insulin resistance is not a consequence of hepatic steatosis in the core transgenic mice. Although, it might be possible that the insulin resistance in these mice affected the hepatic steatosis, since insulin resistance may be one factor that causes hepatic steatosis [326]. However, the impairment of very-low-density lipoprotein (VLDL) secretion from the liver and hypo-β-oxidation of fatty acids are considered to be the major cause of hepatic steatosis in core transgenic mice [320, 327].

A recent report showed that intra-hepatic expressing of the full-length HCV genome in transgenic mice resulted in impaired clearance of virus-infected hepatocytes [328]. The defect in viral clearance was associated resistance of transgenic hepatocytes to Fas-induce apoptosis, which is of importance for killing by CTLs. This may be a mechanism that contributes to viral persistence and also development of HCC.

Another study showed that expression of HCV proteins in hepatocytes has an influence on the course of viral infection in mice. Transgenic mice expressing the full-length HCV genome were more susceptible to viral-induced hepatitis than control mice. The

explanation for the more severe hepatitis and the increased mortality in the transgenic mice was suggested to be due to the increased viral replication in liver cells and thereby an increased CTL-mediated lysis of infected hepatocytes [116]. Importantly, in the same study, they found a functional inhibition of IFNα-induced signaling through the Jak-STAT (signal transducer and activator of transcription) pathway in the liver of transgenic mice *in vivo* [116, 329] and *in vitro* [330]. *In vivo* experiments showed that cells expressing viral proteins had a lower amount of phosphorylated STAT1 in the nucleus. This may decrease the binding of STAT transcription factor complexes to their cognate response elements in the promoters of IFN-stimulated genes (ISGs). Subsequently, this results in an inefficient activation of these genes. Such a mechanism may promote persistence of HCV Moreover; it may also contribute to a reduced responsiveness to IFN-based therapies [116, 329]. It has recently been reported that HCV NS3/NS4A protein *in vitro* interferes with and block the phosphorylation and effector function of interferon regulatory factor-3 (IRF-3). This will also prevent IFN signaling, and thereby promote viral persistence [43]. It has also been proposed that NS5A or E2 proteins can interfere with the IFN-induced antiviral effector protein RNA-dependent protein kinase (PKR) [47, 50, 51].

Several of the produced transgenic lineages do not show any overt pathology at all [308-311, 313-315], suggesting that some of the HCV proteins are not direct cytopathic for the liver. It is also possible that the proteins must be expressed in a certain way or during certain conditions that we do not know today. Importantly, a few or multiple HCV proteins may be involved in regulatory mechanisms contributing to immune escape and viral persistence. It will be of crucial importance to further characterize the HCV involvement in modulating the host immune response and their functions.

By summarizing all data from the transgenic mice models available today only a few has succeeded in confirming the presence of the HCV proteins (i.e. core, E1, E2, and NS5A) *in vivo* by either western blot or by immunohistochemistry. Therefore, it is of crucial importance to develop new transgenic mice lineages expressing the p7, NS2, NS3, NS4A, NS4B, and NS5B that may help to characterize the different HCV proteins.

Mouse livers repopulated with human hepatocytes

Mice that over express a urokinase plasminogen activator (uPA) transgene from the albumin (Alb) promoter experience hepatocellular atrophy starting at birth when the

albumin promoter becomes active [331]. Homozygous animals (Alb-uPA) can be rescued by transplantation of normal hepatocytes, which undergo rapid proliferation to replace dying hepatocytes [332]. By combing these Alb-uPA transgenic mice with immunodeficient SCID mice, it was possible to generate a model system that allows for orthotopic engraftment of human hepatocytes. This microenvironment was suitable for engraftment and expansion of transplanted human hepatocytes and the reconstitution with human hepatocytes was approximately 50% of the liver cell mass. Recent studies showed that human hepatocytes repopulated within mouse livers could be infected by HBV [333] and by HCV [334]. Inoculation with serum from patients with HCV resulted in persistent HCV viremia in about 75% of the mice with high-level human hepatocyte engraftment. The hepatocytes survived for up to 35 weeks, the mice experienced high levels of viremia (viral titers similar to those in infected humans), and the virus can be serially passed to new mice. This provides a convincing evidence for active replication and production of infectious viral progeny in this system [334]. This model system allows studying several unique and important features of HCV. First, HCV infection and replication occur in hepatocytes and in its natural environment. Second, infections can be achieved with viral strains isolated from different HCV-infected donors. Third, it allows for studies on the natural infection process with an easily measurable readout. Most importantly, this model system may be used for the development and evaluation of new antiviral compounds. In addition, this system will also allow for evaluation of passive immunization schedules and testing of compounds that block viral entry. However, it should be noted that this model also has some limitations. In this model the mice lack a functional immune system, which is known to play a central role in the pathogenesis during HCV infections. Therefore, the model in its present form is not suitable to study active vaccine strategies. Another limitation is ease and accessibility of this model, since these animals are sensitive and affected by bleeding disorders and severe immunodeficiency (approximately 35% mortality in newborns). Also the limited access to fresh human hepatocytes is a clearly limitation.

AIMS OF THE STUDY

The aims of this thesis were to develop vaccines and experimental models, and to study and characterize the humoral and cellular immune responses targeted against the HCV NS3/4A protein.

1. To characterize the humoral and CD4+ T cell responses against HCV NS3/4A in mice after protein and genetic immunization **(Paper I)**

2. To understand the importance of NS4A for the immunogenicity of NS3 as a genetic vaccine **(Paper II)**

3. To enhance the immunogenicity of HCV NS3/4A-based vaccines by modifying the gene and protein expression **(Paper III)**

4. To generate a HCV NS3/4A-transgenic mouse **(Paper IV)**

COMMENTS ON MATERIALS AND METHODS

MICE

Inbred mice of different genetic backgrounds (BALB/c (H-2^d), C57BL/6 (H-2^b), and CBA (H-2^k)) were used throughout the study. The mice were obtained from the breeding facility at Karolinska University Hospital, Huddinge, Microbiology and Tumor Biology Center, Karolinska Institutet, or commercial vendors. Mice transgenic for the HCV NS3/4A protein were generated at the Unit for Embryology and Genetics at Karolinska Institutet. All animal experiments were approved by the Ethical Research Committee at Karolinska University Hospital.

RECOMBINANT AND PEPTIDE ANTIGENS

The recombinant NS3 (rNS3) protein was kindly provided by Darrell L Peterson, Department of Biochemistry, Commonwealth University, VA, USA. The NS3 protein (not including NS4A) was produced in *Escherichia coli* as previously described [335].
An H-2^b restricted NS3-specific CTL epitope was identified from a set of 20 amino acids (aa) long overlapping synthetic peptides spanning the complete NS3/4A region. The 20 aa peptides were assayed for stabilization of surface expression of MHC class I molecules on transporter associated with antigen processing (TAP) 2 deficient RMA-S cell line [336, 337]. By this approach one 20-mer long peptide was identified that bound H-$2D^b$ molecules with seemingly high affinity. To determine the optimal length of the peptide, nine aa long peptides, with an eight aa overlap, were synthesized and evaluated for H-$2D^b$ binding. We were able to identify one candidate peptide (sequence GAVQNEVTL), located at the C-terminal domain of NS3, 21-amino acids from the NS3/4A junction. This epitope was used for detection of NS3-specific *in vitro* CTLs and quantification of the number of NS3-specific CD8+ positive cells after vaccination with NS3-based vaccines.

DNA VACCINE ANTIGENS AND VIRAL VECTORS

A full length wildtype (wt) NS3/4A gene from a HCV genotype (gt) 1 infected patient was amplified using primers flanking the start of NS3 and end of NS4A previously

described by Zhang *et al* [338]. The wtNS3/4A gene fragment was cloned into different vector backbones i.e. pcDNA3.1, pSecTag2, and pVAX1 (Invitrogen, San Diego, CA, USA). The NS3 protein was chosed, based on the arguments raised in the introduction, which in brief were that it is a large protein which decreases the risk of genetic non-responder status on T-cell level, the immune response to NS3 has been found to be of importance, it has a limited variability [339, 340], and that the immune responses are cross reactive between different genotypes [87, 88, 341, 342]. NS4A was included in the NS3-based vaccines since NS4A is required to generate the complete and functional NS3 protease. To determine the importance of NS4A in NS3-based vaccines, two vaccine candidates were generated, one only encompassing the NS3 gene (wtNS3), and another candidate with a disrupted cleavage site between NS3 and NS4A, generating a NS3/4A fusion protein (mNS3/4A). To enhance the translation efficiency and immunogenicity of the wtNS3/4A gene, a synthetic NS3/4A vaccine candidate was generated in which the codon usage was optimized (co) for human cells. Another approach to enhance expression levels and possibly also the immunogenicity was mRNA amplification using the Semliki forest virus (SFV) replicon. The wtNS3-, wtNS3/4A-, and mNS3/4A-genes were isolated by polymerase chain reaction (PCR) and packaging of recombinant RNA into recombinant (r) SFV particles was done using a two-helper RNA system [343-345].

IMMUNIZATION PROTOCOL

Mice were immunized with NS3-based vaccines, as DNA plasmids, proteins, peptides or rSFV particles to generate an immune response against the vaccine antigen. DNA plasmids expressing the wtNS3-, wtNS3/4A-, mutNS3/4A-, and coNS3/4A-genes were administered intramuscularly (i.m.) by needle injections into the tibialis anterior (TA) muscles using 10 to 100µg DNA/mouse. In some experiments, the TA muscles were pre-treated i.m with 50µL 0,01mM cardiotoxin (CT; Latoxan, Rosans, France) prior to DNA immunizations. Five days after CT treatment, regenerating TA muscles were immunized. DNA plasmids were also immunized transdermally using the gene gun apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Transdermal immunizations using a 4µg DNA dose were performed on shaved mouse abdomens. Protein and peptide immunization was performed with different doses and adjuvants (i.e. complete Freunds adjuvants (CFA) or incomplete Freunds adjuvants (IFA)). Protein and peptides were given i.m., intraperitoneal (i.p.) or subcutaneous (s.c.) in the base of the tail.

Immunization with rSFV particles was given s.c. in the base of the tail using 1 x 10^7 virus particles/dose and time point. In indicated experiments, mice were boosted with the same dose and route at monthly intervals.

IN VIVO TUMOR CHALLENGE

In vivo challenge using tumor cells expressing the desired viral antigen after vaccination using the same antigen is one way to investigate how effective, and specific, vaccine-primed CTLs are in preventing growth of tumor cells. The *in vivo* tumor challenge model used here was based on previously described protocol by Encke *et al* [268]. First we generated a wtNS3/4A-expressing Sp2/0 myeloma (H-2^d) cell line and a wtNS3/4A-expressing EL-4 lymphoma (H-2^b) cell line. Second, we optimized the protocol regarding antigen dose, administration route, and number of tumor cells inoculated. The design of the tumor model is outlined in figure 9. The number of tumor cells inoculated was chosed from the number that generated tumor growth in all MOCK immunized mice. The experimental design was to immunize mice (groups of 10 mice) intramuscularly (i.m), transdermally by gene gun or subcutaneously (s.c.) with different immunogens. Mice were immunized once, twice or thrice with four weeks intervals, and two weeks after the last immunization tumor cells (1 x 10^6 cells/mouse) were inoculated in the right flank of the mouse. The tumor model was also used for therapeutic vaccination studies were we first inoculated tumor cells and after 6 or 12 days immunized mice with the viral antigen. The kinetics of the tumor growth was measured through the skin using a slide caliper at days six to 20 after tumor inoculation. The tumor growth was measured blinded for the investigator and the same person did all measurements every second or third day. The protection against tumor growth in the different groups was then compared statistically using the area under the curve (AUC) and analysis of variance (ANOVA) test.

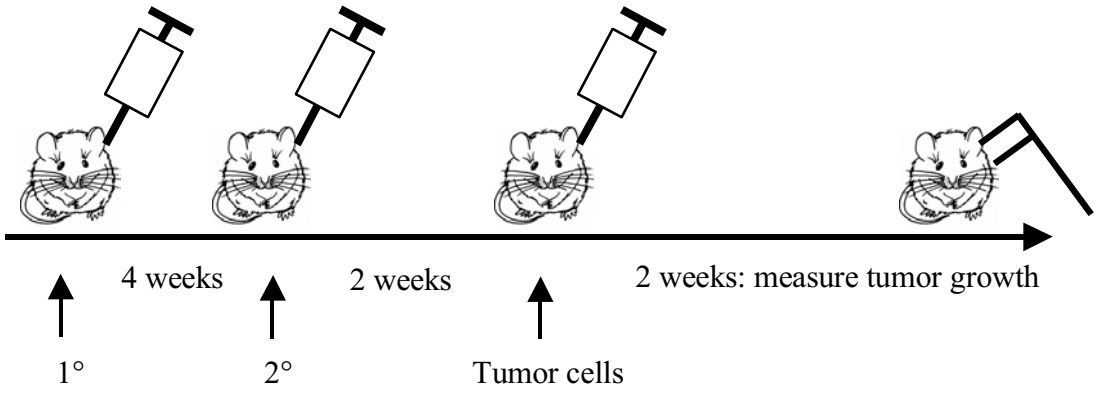

Figure 9. Schematic illustration of the tumor model experimental design.

DETECTION AND QUANTIFICATION OF CYTOTOXIC T CELLS

To measure the lytic activity of vaccine primed cytotoxic T cells (CTLs) a standard ^{51}Cr-release assay was used. In this method, precursor CTLs, present in the spleen of immunized animals were expanded *in vitro* in presence of a MHC class I restricted peptide or transfected cell lines expressing the desired antigen. After *in vitro* re-stimulation, the ^{51}Cr-release assay is set up. Target cells are either peptide coated or transfected cells, which are labeled with radioactive ^{51}Cr and incubated with effector cells for four hours. Killing is measured indirectly by detecting ^{51}Cr released by lysed target cells into the supernatant using a γ-counter. One should keep in mind that peptide specific CTLs can be primed *in vitro* and it is therefore important to use relevant controls, such as re-stimulation with unrelated peptide or transfected cell lines. By using this approach we will get naturally processed MHC class I restricted peptides presented for the effector cells on MHC class I. It is worth noting that the use of transfected cells will generally result in a lower lytic activity compared to when using peptide coated target cells. This is most probably due to the lower number of antigen specific peptides in MHC class I molecules on the transfected target cell, compared to a target cell loaded with peptides exogenously. Although, transfected target cells will mimic the *in vivo* situation, it is sometimes a better alternative to use peptide coated target cells, especially when the differences are small in lytic activity between different groups.

To avoid potential artifacts due to in vitro re-stimulation it is also of importance to quantify the number of antigen-specific CTLs directly *ex-vivo*. Direct *ex-vivo* quantification can be analysed by different techniques, such as by flow cytometry using

tetrameric MHC molecules, tetramers [346], or dimeric MHC-Ig fusion proteins [347]. It is also possible to quantify the number of antigen specific CTLs by ELISPOT assays originally developed in the 1980s [348, 349]. In our studies the MHC-Ig technique was used to quantify the frequencies of peptide specific CD8+ T-cells. MHC-Ig fusion proteins consists of one MHC class I molecule, which is fused to the variable domain of an immunoglobulin molecule (Figure 10). The MHC-Ig fusion protein is produced in eucaryotic cells and during intracellular transport the MHC binding groove is filled with cell-derived peptides. One advantage is that prior to use, the MHC-Ig fusion protein can be loaded with the desired peptide. Loading of a specific peptide into the binding groove of the MHC-Ig molecule is facilitated by the presence of excess peptide of interest. One disadvantage of this technique is the rather high background staining due to binding to Fc receptors. However, this background may be reduced by pre-incubating the cells with an antibody blocking the Fc receptors prior to addition of the fusion protein. It should be noted that the MHC-Ig technique only offers a quantitative analysis and do not provide any information about the functionality of the T cell. Thus, it is important to verify the results obtained with MHC-Ig by other functional assays such as ^{51}Cr-release assay or staining of intracellular markers (i.e. perforin and granzyme) intimately linked with cytolytic activity.

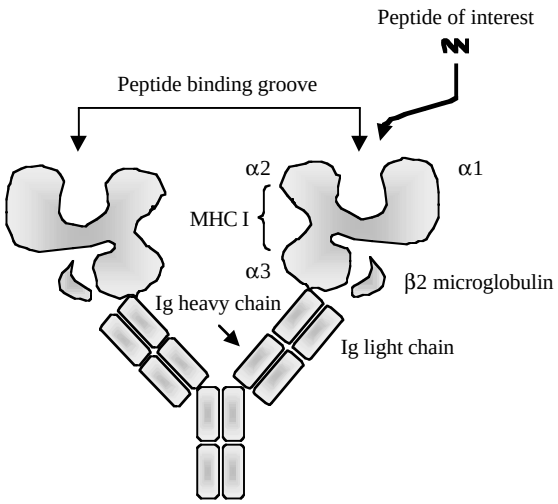

Figure 10. Schematic representation of the MHC class I:Ig dimeric protein.

PRODUCTION OF TRANSGENIC MICE

Due to the lack of infectious small animal models and efficient cell cultures systems to study HCV infection transgenic mice expressing HCV protein will serve as important tools. We therefore generated a transgenic mouse lineage with an intra-hepatic expression of the NS3/4A protein. The developmentally regulated mouse major urinary protein (MUP) promoter was used to target expression to the liver. This promoter is activated 14 days after birth. The full-length wtNS3/4A (genotype 1) gene was cloned into the pMUP expression plasmid used for microinjection. The transcriptional unit was microinjected into fertilized oocytes from C57BL/6 (H-2^b) x CBA (H-2^k) mice using standard techniques. Transgenic mice were identified by PCR and for protein expression by western blot.

STATISTICAL ANALYSIS

Fisher's exact test was used for frequency analysis and Student's t-test and Mann-Whitney U-test was used for comparing mean values between groups. Kinetic tumor development in two groups of mice was compared using the area under the curve (AUC). AUC values were compared using analysis of variance (ANOVA). The calculations were performed using the Macintosh version of the StatView software (version 5.0).

RESULTS

CHARACTERIZATION OF HUMORAL AND TH-CELL IMMUNE RESPONSES TO THE NS3/4A PROTEIN IN MICE IMMUNIZED WITH NS3 AS PROTEIN OR DNA (PAPER I)

In the first study we characterized the humoral and Th-cell immune responses to NS3 as a recombinant (r) protein or DNA immunogen in mice of different genetic background and H-2 haplotype (H-$2^{k, d, b}$), and by different routes and adjuvants. We found that rNS3 protein used in combination with adjuvants was superior to wtNS3/4A as a DNA immunogen regarding priming of a stronger humoral immune response. The wtNS3/4A DNA-based vaccines did induce an antibody production, but not in the same magnitude as rNS3 protein. However, when using rNS3 protein without adjuvants, 1-10μg NS3 protein induced comparable antibody titers as 100μg wtNS3/4A DNA. Thus, the DNA-mediated protein expression after *in vivo* immunization equals 1-10μg of exogenous rNS3 protein. Mice immunized with rNS3 protein (with or without adjuvant) primed a predominant Th1/Th2-like response, characterized by a mixed (i.e. IgG1, IgG2a, IgG2b, and IgG3) antibody production. In contrast, wtNS3/4A DNA immunized mice induced a strong Th1-like profile, characterized by an IgG2a and IgG2b antibody production. Exogenous NS3 in adjuvant primed NS3-specific CD4+ T-cells more efficiently and more rapidly as compared to wtNS3/4A DNA. Our results shows that wtNS3/4A given as DNA immunogen effectively primes CD4+ Th1-like immune responses, whereas rNS3 protein primes a mixed CD4+ Th1/Th2-like profile regardless of route, dose or adjuvant.

THE IMPORTANCE OF NS4A IN NS3-BASED VACCINES (PAPER II)

In the second study we analyzed the importance of NS4A in NS3-based genetic vaccines. We compared different NS3-based genetic vaccines, using different administration routes and doses with respect to protein expression levels, humoral, CD4+ and CD8+ T-cell responses, and the ability to prevent *in vivo* growth of tumors expressing the wtNS3/4A protein. The use of vaccines encompassing both NS3 and NS4A was superior to vaccines containing only NS3. By examination of several NS3-

based vaccine candidates we could show that a functional protease was important to maintain the enhanced immunogenicity. We could also show that the increased immunogenicity was not just due to the addition of new CD4+ Th-cell epitopes within NS4A [341, 350]. These results were confirmed by expressing the different NS3-based genetic vaccines using the Semliki forest virus (SFV) vector based expression system. In this system, the expression of the functional wtNS3/4A gene showed the highest expression levels. This suggests that superior expression levels by the NS3/4A gene probably explain the more potent immune activation. The wtNS3/4A plasmid induced a typical Th1-like profile, assessed by a high IgG2a/IgG1 ratio, whereas wtNS3 induced a mixed Th1/Th2-like phenotype. The NS3/4A gene activated cellular response more efficiently than the NS3. The number of NS3-specific CD8+ T-cells after immunizing mice using NS3/4A was approximately 2-4% of the total CD8+ T-cell population. The wtNS3/4A plasmid induced more efficient CD4+ T-cell priming in mice, and inhibited wtNS3/4A-expressing tumor cells in immunized and challenged mice more efficiently than the wtNS3 gene alone. Protection was also maintained when immunizing with a low dose of wtNS3/4A but not when immunizing with a lower dose of wtNS3. Both intramuscular and transdermal administration of the wtNS3/4A vaccine using different doses protected against *in vivo* tumor challenge. In conclusion, NS3/4A was found to be a more potent than NS3 in priming *in vivo* functional immune responses.

MODIFICATIONS AND OPTIMIZATION OF THE NS3/4A GENE TO ENHANCE THE IMMUNOGENICITY OF THE GENETIC VACCINES (PAPER III)

In this study we investigated if the immunogenicity of NS3/4A could be further enhanced by codon optimization or mRNA amplification using the Semliki forest virus (SFV) system. Codon optimization (co) clearly generated higher protein expression levels in comparison to the wildtype (wt) NS3/4A gene. Both codon optimization and mRNA amplification using SFV enhanced the immunogenicity with respect to humoral immune responses as compared to wtNS3/4A. The IgG subclass distribution was not changed as a result of codon optimization. Both wt- and coNS3/4A showed a distinct Th1-like profile. However, when immunizing transdermally using gene gun we observed a skewing towards a mixed Th1/Th2-like phenotype. The codon-optimized gene primed a higher frequency of NS3-specific CD8+ T-cells as compared to wtNS3-DNA. We were able to prime *in vitro* detectable CTLs with a lytic activity using all of

the NS3-based genetic vaccines. The highest *in vitro* lytic responses were primed by coNS3/4A and wtNS3/4A-SFV supporting that these vaccines have an enhanced immunogenicity. We could show that only mice immunized with NS3-based genetic vaccines were protected against challenge with tumors expressing wtNS3/4A. The CD8+ T-cells are the key T-cell population in protection against tumor growth. The protection was shown to be independent of both B-cells and CD4+ T-cells. When immunizing C57BL/6 mice with a single dose we found that only vaccines containing NS3 plus NS4A generated protection against tumor challenge. Mice immunized with rNS3 protein, an NS3-derived MHC class I-binding peptide, or an empty vector did not confer protection. Finally, we could show that therapeutic vaccination using the coNS3/4A vaccine construct induced a reduction in tumor growth, when immunized six to twelve days after tumor inoculation. Thus, both codon-optimization and mRNA amplification by SFV effectively enhances the immunogenicity of NS3-based genetic vaccines.

CHARACTERIZATION OF THE *IN VIVO* EFFECT OF INTRA-HEPATIC EXPRESSION OF THE NS3/4A PROTEIN IN TRANSGENIC MICE (PAPER IV)

In the fourth study we characterized the *in vivo* effect of wtNS3/4A-protein expression in mouse livers by generating a new transgenic mouse lineage. In these transgenic mice the expression of the wtNS3/4A protein was liver specific. The expression levels of NS3 ranged from 0,1 to 8 μg per g liver tissue. We could also confirm NS3 protein expression in mice livers by immuno histochemistry. The staining pattern in the transgenic mouse hepatocytes with a cytoplasmic distribution of the NS3 protein was similar to the staining observed in chronic infected human livers [351]. We also noted that the transgenic livers were slightly enlarged and the number of nuclei per standardized liver area was less in the transgenic mice compared to the non-transgenic mice, indicating enlarged liver cells in the transgenic mice. However, the body weight did not differ between transgenic and non-transgenic mice. Histological and biochemical analysis of the transgenic mice did not reveal any overt liver pathology due to the wtNS3/4A protein expression. Interestingly, we did observe less spontaneous appearing intra-hepatic inflammatory foci in the transgenic mouse livers as compared to age-matched controls. Thus, intra-hepatic expression of wtNS3/4A does not seem to cause a

readily detectable liver pathology but may affect the distribution of immune cells within the liver.

DISCUSSION

The HCV NS3 protein is a multifunctional enzyme complex that performs several important functions in the viral life cycle. The NS3 protein participates in un-winding and strand separation of the viral RNA, and proteolytic processing of the viral polyprotein. The performance of these key enzymatic functions most likely explain why the NS3 region is a genetically stable region and well conserved among different genotypes. Also, several groups have shown that strong NS3-specific CD4+ and CD8+ T-cell responses are important for resolution of acute infections, and in chronic infections during antiviral therapy [87, 88, 176, 177]. Recent studies in chimpanzees also support the notion that vigorous and multispecific Th1-type NS3-specific immune responses seems to be of importance [284, 285]. Thus, several factors therefore favor the use of NS3 as a possible target for antiviral drugs and vaccines.

Most previous studies on NS3 as a genetic vaccine have used the NS3 gene alone, or parts thereof [268, 269, 281, 352, 353]. However, one should remember that the complete functional NS3-protease requires the NS4A protein. In fact, NS4A has been shown to be essential both for the protease and for the NTPase/helicase activity. This may be explained by the observations that NS4A can enhance the intracellular stability of NS3 and that NS4A targets the NS3/4A-complex to intracellular membranes. The latter is thought to be essential for the formation of the replication complex [39]. We therefore included NS4A in our NS3-based vaccines.

We noted early on when using NS3-based genetic vaccines that our DNA plasmids induced 10- to 100-fold higher antibody titers compared to other reports [268, 269, 353]. This may be explained by several factors, as for example different vector backbones and slightly different modes of immunization. However, it could also be explained by the simple fact that we had included NS4A in our plasmids **(Paper I and II)**. As previously mentioned, the inclusion of NS4A in NS3-based vaccines might increase the intracellular stability of NS3 and/or target NS3 to intracellular membranes (i.e. ER). These properties of the complete protease complex might in turn have influenced the intrinsic immunogenicity of NS3. To sort this out we analyzed different types of NS3-based vaccines, given at different doses and through different administration routes, and in different compositions (i.e. recombinant protein, DNA, self-replicative viral vectors). We found that recombinant NS3 protein, given with or without adjuvant, primed the highest and most rapid humoral immune response as compared to the

genetic vaccines. However, the recombinant protein primed a Th2-like immune response. This is generally not preferred when aiming towards a vaccine against a viral infection. In contrast, it is thought that a strong Th1-like profile may promote the activation of anti-viral CD8+ CTLs [354]. We could show that the most potent and effective humoral immune response with a Th1-like profile was generated when NS4A was included in the vaccine **(Paper I, II, and III)**. In addition, codon optimization or mRNA amplification using SFV further enhanced the humoral immune responses, both with respect to speed and magnitude. Both codon optimization and mRNA amplification using SFV increased the expression levels of NS3, which we believe, at least partly, may explain the enhanced immunogenicity of the vaccine. However, others did not find that immunization with SFV particles enhanced the NS3-specific immune response [281]. This may be explained by the fact that we included a translation enhancer in our vaccine candidate which may enhance the expression levels by approximately ten times [344, 355]. In addition, our NS3-SFV vaccine also contained NS4A, which in our hands enhanced the expression levels of NS3. In fact, all vaccine constructs including NS3 and NS4A primed a more rapid and stronger humoral immune response as compared to vaccines containing NS3 alone. We could also show that to fully utilize the benefit of including NS4A in NS3-based vaccines, a functional cleavage site between NS3 and NS4A seemed to be of importance. The addition of NS4A to the NS3-based vaccines did not add any new CD4+ Th epitopes that could explain the enhanced immunogenicity of NS3. Also, the wtNS3- and wtNS3/4A-plasmids activated B-cells *in vitro* at an equal level suggesting that no new immune stimulation motifs had been introduced. Thus, the enhanced immunogenicity is most likely due to the enhanced expression levels of the NS3-protein conferred by the inclusion of NS4A.

By analyzing the IgG subclass distribution after immunization with different NS3-based vaccines, we could indirectly study the Th1- and Th2 profiles of the primed T-cell responses. In mice it is known that high levels of antigen-specific IgG1 indicate a Th2-like response, whereas high levels of antigen-specific IgG2a signal a Th1-like response. Thus, we could compare IgG2a/IgG1 ratios primed by the different immunogens. We found that immunization with recombinant NS3 protein, with or without adjuvant, primed a low (<1) IgG2a/IgG1 ratio suggesting a Th2-like response. In contrast, wtNS3, wtNS3/4A, and coNS3/4A as DNA-vaccines given intramuscularly, and wtNS3/4A-SFV given subcutaneously, predominantly primed Th1-like responses. Surprisingly, the presence of NS4A even further skewed the T helper profile towards a Th1-like phenotype. This further supports the inclusion of

NS4A.in NS3-based genetic vaccines. In agreement with previous reports did we also note that the administration route was a key factor in determining the Th-phenotype [213, 356-358]. Intramuscular immunization using DNA-vaccines primed a Th1-like profile, whereas transdermal gene gun immunization primed a subclass ratio suggesting a mixed Th1/Th2 response **(Paper I, II, and III)**. The skewing towards a mixed Th-phenotype by immunizing transdermally using the gene gun may possibly be explained by that the gene gun can deliver the DNA directly into the cells. This could bypass any effects of Th1-promoting *CpG* motifs in the DNA plasmid backbone. It has recently been shown that interactions between the Toll like receptor (TLR) 9 on the surface of an APC and plasmid *CpG* motifs may promote release of proinflammatory Th1-type cytokines [359]. It is also possible that different delivery routes may target different subsets of DCs, which in turn preferentially may prime either Th1- or Th2-responses. Additionally, the nature of the antigen may skew the type of Th-response. These factors need to be studied in a greater detail.

The cellular immune responses primed by the different NS3-based vaccines were evaluated by assessing the NS3-specific CD4+ and CD8+ T-cell responses. The complete protease complex delivered as the wtNS3/4A-DNA plasmid primed proliferative CD4+ Th-cell response more efficiently than plasmids containing wtNS3 alone. However, the priming of CD4+ Th-cell responses was even more potent and rapid when using rNS3 protein. However, consistent with the IgG subclass pattern did the recombinant protein prime a mixed Th1/Th2 CD4+ T-cell response as determined by *in vitro* cytokine production. In contrast, as previously observed regarding the IgG subclasses did the wtNS3/4A-DNA vaccine prime a more Th1-like CD4+ T-cell cytokine response **(Paper I and II)**. Taken together, these data favor the use of wtNS3/4A-DNA as a vaccine candidate, when aiming at inducing a Th1-like CD4+ T cell response against HCV NS3. These findings support and extend previous observations suggesting that the NS3 gene alone, given as a DNA vaccine or as a self-replicating RNA vaccine, preferentially primed Th1-like responses [268, 352, 353]. Thus, in contrast to exogenous protein antigens, does genetic vaccines seem to favor the priming of NS3-specific Th1-like responses.

The CD8+ T-cell responses were examined by several approaches, by analyzing the *in vitro* lytic activity of NS3-primed T-cells, by a direct *ex-vivo* quantification of NS3-specific CD8+ T-cells, and by the ability of primed T-cells to inhibit NS3/4A-tumor growth *in vivo*. All genetic vaccines containing both NS3 and NS4A primed CTLs with higher *in vitro* lytic activity against peptide coated or stably transfected target cells, as

compared to vaccines containing NS3 alone. Thus, our results argue that the inclusion of NS4A in NS3-based vaccines enhances priming of NS3-specific CTLs. This would suggest that increased expression levels of NS3 favor the induction of a potent NS3-specific CD8+ T-cell response. This was actually further confirmed, when we could show that an even higher lytic activity could be obtained when using the coNS3/4A-DNA or wtNS3/4A-SFV vaccines. Thus, in our hands does increased expression levels favor strong NS3-specific CTL responses.

As a technical note, when using stable transfected cells as targets the lytic activity seemed to be lower as compared to when using peptide coated target cells. This is most probably due to a less dense presentation of peptide-loaded MHC class I molecules on the transfected cell. Thus, when comparing immunization schedules where the differences in priming may be quite small the most sensitive assay, i.e. peptide coated target cells, should be used. However, the use of transfected target cells has to be seriously considered since this more accurately mimics the *in vivo* situation, with respect to the processing and presentation of viral peptides.

The number of NS3-specific CD8+ T-cells was analyzed by direct *ex-vivo* flow cytometry. The percent CD8+ T-cells specific for the H-2D^b-restricted NS3 peptide, primed by wtNS3-DNA, wtNS3/4A-DNA, coNS3/4A-DNA or wtNS3/4A-SFV immunization, ranged from 0,3 to 4% of the whole splenic CD8+ population. However, the percent NS3-specific CD8+ T-cells was higher after immunization using the coNS3/4A-DNA compared to wtNS3-DNA, supporting the observation that higher expression levels of NS3 primes a stronger CD8+ T-cell activation **(Paper II and III)**.

We also achieved an effective priming of NS3-specific CTLs by low doses of DNA using transdermal administration. Low immunogenic doses of DNA are of crucial importance when going forward with a potential therapeutic vaccine to higher animals. In fact, similar doses of DNA to those used by us have already been successfully used in human trials where the DNA vaccine was capable of generating a protective humoral and cellular immune responses [224].

One additional approach to examine the potency of genetic vaccines is to test these by *in vivo* models. We choose to use an *in vivo* model based on the inhibition of tumor growth. In this model, the potency of the vaccine-primed NS3-specific T-cells is evaluated by a challenge with wtNS3/4A-expressing tumor cells. The level of protection is monitored kinetically by measuring the growth of the solid tumor through the skin. Protection against tumor growth was vaccine specific and the cell population responsible for the protection was found to be CD8+ T-cells. A striking improvement

in the protection against tumor challenge was seen when NS4A was included in the NS3-based vaccines. First, only mice immunized using the gene gun with DNA vaccines containing both NS3 and 4A were protected against tumor growth. Second, consistent with previous data reported herein, was the protection superior in groups vaccinated with vaccines having a high level of NS3 expression (i.e. coNS3/4A). Third, we could show that protection could be obtained with a tenfold lower DNA dose given i.m. when using the wtNS3/4A-DNA vaccine. This was not the case when using wtNS3 gene alone. Thus, the results in the tumor-inhibition model were perfectly in agreement with data from the *in vitro* studies of the NS3-specific responses.

As previously mentioned, protection on the tumor-inhibition model was conferred by specific CD8+ CTLs. We found that intramuscular, subcutaneous, or transdermal immunization schedules using NS3-based genetic vaccines induced a comparable level of protection. The latter observation is a bit surprising since we could show that transdermal immunization preferentially primed a Th2-like CD4+ T cell response. Thus, this Th2-like response did not interfere with effective priming of neither *in vitro* nor *in vivo* detectable CTLs. Moreover, using the gene gun the protection against tumor growth and priming of NS3-specific CD8+ T-cells was independent of both CD4+ T-cells and B-cells. We were unable to prime protection against tumor growth using rNS3 protein, NS3-specific MHC class I peptides, plasmid expressing irrelevant protein, or empty plasmid **(Papers II and III)**. Thus, an effective priming of *in vivo* protective CTL responses was independent on specific T helper function but required endogenous expression of the vaccine gene. The T helper-independent priming of NS3/4A-specific CD8+ CTLs should be further investigated, in particular with respect to long-lived memory CTL responses. Such responses may require the priming of specific T help [360].

We found early on that protection against tumor growth seemed to be easier to obtain in C57BL/6 mice as compared to BALB/c. In C57BL/6 mice a strong protection was seen already after a single genetic vaccine dose, whereas a single dose only conferred a partial protection in BALB/c mice. Therefore, to analyze the differences in intrinsic immunogenicity of NS3-based immunogens, the H-2^b mice may be to sensitive, and therefore H-2^d mice might be preferred. Thus, both these strains are of value in studying the various responses in detail.

When aiming at the development of a therapeutic vaccine it is important to examine the efficacy of the genetic vaccine in a therapeutic immunization regimen. This was explored by first inoculating the mice with tumor cells expressing wtNS3/4A, and then at later time points vaccinate the same mice therapeutically. This could show if the

primed CD8+ T-cells were able to reduce, or even halt, tumor growth. In fact, mice immunized transdermally with coNS3/4A-DNA six to twelve days post tumor inoculation did show a statistical significant reduction in tumor sizes, indicating a fast and effective priming of functional CD8+ T-cells **(Paper III)**. Thus, our NS3/4A-based vaccines effectively activate both CD4+ and CD8+ T-cells against NS3. These T-cells, mainly CTLs, infiltrate tumors expressing NS3/4A-protein and confers protection against tumor growth *in vivo.*

A major concern in genetic vaccine development is that the expressed vaccine gene has toxic or otherwise unwanted properties. One way to address this potential problem is to express the gene in various tissues and look for toxicity. A possibly even better approach can be to express the gene *in vivo*. Such an *in vivo* expression model could also serve to evaluate the efficacy of the vaccine. The *in vivo* functional effects and properties of NS3/4A were therefore investigated by generation of a new transgenic mouse lineage with an intra-hepatic expression of the NS3/4A-protein. The protein expression was controlled by and targeted to the liver using the MUP promoter. Protein expression was detected by western blot and by immunohistochemistry. The protein expression levels of 0,1 to 8 μg per g liver tissue were comparable to other transgenic mouse lineages generated using the MUP promoter (David Milich, personal communication). We observed that NS3 expression was restricted the cytoplasm of mouse hepatocytes. Thus, the pattern of NS3/4A expression in the transgenics is fully comparable to that seen in infected humans [351]. A staining pattern of the transgene protein consistent with HCV infected humans has also been reported for core, E1, E2, and NS5A in other transgenic models [116, 118-120, 308, 311-313]. Importantly, only one other non-structural HCV protein has so far been detected *in vivo* in transgenic mice, despite that mRNA has been detected by PCR [314]. To our surprise, the NS3/4A-transgenic mice did display slightly (around 10%) enlarged livers as compared to age-matched control mice. This enlargement seemed to be due to an increased size of the hepatocytes since the number of nucleus per standardized surface area was lower in the transgenics. The increased size of hepatocytes may possibly be due to an accumulation of fat or glycogen. However, this needs to be thoroughly investigated. We did not see any obvious changes by histology that could explain this enlargement. However, it is possible that this 10% difference was too small to be detected by histology.

Some of the other HCV transgenic mouse lineages, in particular those expressing core alone or in combination with other HCV proteins, display liver pathology at early age. This appears as accumulation of fat deposits within the hepatocytes. Some lineages

even develop HCC. It is important to note that all HCV transgenic mouse models available today are based on transgenes of genotype 1. However, the HCV infected patients, in whom the development of steatosis seems to be related to the virus, are most often infected by HCV of genotype 3 [110-115]. Also, it is important to note is that not all of the HCV transgenic mice that are transgenic for the same HCV protein develops the same type of pathology. This may be due to the use of mice with different genetic background, insufficient levels of protein expression, transgene integration site, or the type of promoter used. These somewhat contradictive results emphasize that findings in transgenic mouse models should be interpreted with care. Anyway, these models may still contribute with important information regarding the *in vivo* effect of the studied protein.

Both biochemical and histological analysis of the NS3/4A-transgenic mice did not suggest any development of overt liver pathology, despite the slight enlargement of the livers. One interesting small phenotypic change was noted. The NS3/4A-transgenic mice had less spontaneously appearing intra-hepatic inflammatory foci. Such foci are commonly found in laboratory mice. Thus, it seems that the liver restricted protein expression of NS3/4A does not lead to a direct induction of overt liver disease or pathology. However, the NS3/4A-complex may possibly affect the distribution of immune cells within the liver **(Paper IV)**. This needs to be further investigated.

In conclusion, these new NS3/4A-transgenic mice may serve as an additional model system to study HCV related disease. Also, they should be valuable in the evaluation of our, and others, newly developed NS3-based vaccine candidates to test if vaccine-primed immune cells can home to the transgenic mouse liver. Finally they should also be useful to study if NS3/4A interferes with the IFN-signaling pathway *in vivo* as recently suggested [43].

GENERAL CONCLUSIONS

*Genetic vaccination using HCV NS3/4A primes CD4+ Th1-like immune responses.

*Inclusion of HCV NS4A in NS3-based genetic vaccines enhances the immunogenicity of NS3.

*Codon optimization and mRNA amplification of HCV NS3/4A further enhances the overall immunogenicity of NS3-based genetic vaccines.

*A new transgenic mouse was generated that express the HCV NS3/4A protein complex in hepatocytes under the control of the MUP promoter.

*Intra-hepatic expression of the HCV NS3/4A-protein does not cause overt liver damage or pathology in NS3/4A-transgenic mice. However, these mice seem to display an altered distribution of immune cells within the liver.

POPULAR SCIENTIFIC SUMMARY

Hepatitis C virus (HCV) is the major cause of chronic liver disease worldwide. Around 170 million people (approximately 3% of the world population) are carriers of the HCV that causes jaundice. Many years of chronic infection (usually more than 10-20) may lead to the development of liver scarring and in the end to liver cancer. Only a small proportion of those infected with HCV clear the infection. Around 60-80% do not clear the infection and become chronic carriers. Today, there is no vaccine available to prevent or cure HCV infections. The current available antiviral therapy for HCV is interferon-alpha and ribavirin. This antiviral therapy can cure 50% of the infected patients. The reason why not all patients respond is probably that the virus continuously changes its appearance and thereby avoids the immune system. These changes are believed to be due to an error-prone enzyme during the viral replication. Some of these variants have been shown to respond less well to antiviral therapy. Today, one of these variants (genotype 1) accounts for approximately 50 percent of all HCV infections worldwide. The outcome of antiviral therapy can therefore quite accurately be predicted by the viral variant infecting the patient. The mechanism of viral persistence is not fully understood, although the high genetic variability is widely believed to play an important role. It is not possible to grow HCV in cell-culture systems, and the only reliable infectious experimental animal model is the chimpanzee. Thus, several factors limit our ability to study the viral life cycle.

We have chosen to study of one the least variable regions of HCV, the non-structural protein 3 and 4A (NS3/4A). This region encodes an enzyme with several important functions in the viral life cycle. Also, genetic changes within this region may lead to loss of the enzymatic activity whereby the virus most likely cannot allow for such mutations. Several reports have shown that patients who clear an HCV infection have a strong immune response, especially T-cells, against NS3. These are likely to be important immune cells for eradicating virus-infected liver cells. In contrast, patients who progress to a chronic infection lack T-cells specific for NS3/4A. This supports the importance of such an immune cell-activation to resolve an HCV infection. Therefore, we investigated the NS3/4A-region as a potential genetic vaccine candidate against HCV infections. We examine this genetic vaccine candidate by vaccinating mice with NS3/4A and evaluated the activated immune responses. We could clearly show that a strong T-cell response against NS3/4A could be activated. We could further enhance this immune response by extensive modifications the NS3/4A gene. A major concern in development of genetic vaccines is that the candidate vaccine has toxic or unwanted properties when expressed in a living organism. We therefore generated a genetically modified mouse that produced the NS3/4A-protein in the liver. This protein production in liver cells occur in HCV infected individuals. This mouse did not develop any obvious liver related disease. In conclusion, we have demonstrated that our vaccine candidate could activate immune responses that are thought to be of importance for clearance of HCV infection in humans. We could also show that the NS3/4A proteins did not seem to cause disease when produced in mouse livers. Thus, the NS3/4A gene is a potential vaccine candidate against chronic HCV infections.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hepatit C virus (HCV) är den vanligaste orsaken till kronisk leversjukdom runt om i världen. Omkring 170 miljoner människor (cirka 3% av jordens befolkning) bär på HCV som orsakar gulsot, men som även kan orsaka levercancer. Av alla som infekteras med HCV så är det bara en liten andel som läker ut infektionen spontant och blir friska. Omkring 60-80% läker ej ut infektionen och blir kroniska bärare av viruset i sin lever. I dags läget finns inget vaccin tillgängligt, men en relativt effektiv behandling finns att tillgå i form av två antivirala medel som heter interferon-alfa och ribavirin. Trots denna behandling så är det nästan hälften av de infekterade patienterna som inte blir botade. Detta beror sannolikt på att viruset kan förändra sitt utseende genom mutationer så att det inte känns igen av kroppens immunförsvar. Denna variation uppstår när virus kopierar sin arvsmassa då detta utförs utan ”korrekturläsning”. Detta leder till att nya varianter ständigt dyker upp som antigen blir ett starkare eller ett svagare virus. Vissa varianter av viruset har visat sig svårare att behandla än andra. Den variant som står för cirka hälften av alla HCV infektioner (genotyp 1) har visat sig vara den mest svårbehandlade. I dagsläget kan man i grova drag förutspå utgången av en behandlig genom att se vilken variant patienten är infekterad med. De bakomliggande orsakerna till varför inte HCV alltid utplånas av kroppens försvar eller av den antivirala behandlingen är till stor del oklara, dock tror man virusets förmåga att mutera spelar stor roll. Man kan idag ej odla HCV i cellkultur och den enda infektiösa djurmodellen som ger reproducerbara resultat är chimpans. Detta medför begränsningar studer av hur virus fungerar.

Vi har valt att studera en av de delar hos viruset som varierar sig minst. Denna del utför flera viktiga enzymatiska funktioner i virusets livscykel. Dessa funktioner är sannolikt orsaken till att mutationer inte förekommer i denna region då dessa skulle kunna medföra att virus dör. Denna del av viruset heter icke-strukturellt protein 3 och 4A (NS3/4A). Det har även visat sig att hos patienter som läker ut HCV infektion spontant eller under antiviral behandling har T-celler riktade mot just denna region. Patienter däremot som inte läker ut HCV infektionen saknar dessa T-celler. Vi har därför valt att använda NS3/4A i en genetisk vaccin kandidat mot HCV. Genom att vaccinera med detta vaccin i möss har vi lyckats att aktivera ett kraftfullt immunologiskt svar mot NS3/4A-regionen. Denna immunaktivering har vi sedan kunnat ytterligare förstärka genom omfattande genetiska förändringar av vaccinet. För att sedan undersöka om NS3/4A har några skadliga effekter, har vi skapat en genmodifierad mus som producerar NS3/4A i levern. En sådan produktion av NS3/4A protein i lever förekommer naturligt under en HCV-infektion hos människa. I denna nya mus kunde vi inte se några direkta skadliga effekter på levern orsakade av NS3/4A-proteinet. Sammanfattningsvis har vi visat att vi via vårt nya vaccin kan aktivera den del av immunförsvaret som är viktig för utläkning av HCV. Det verkar inte som NS3/4A medför några direkt skadliga effekter på celler. HCV NS3/4A är därför en lovande vaccin kandidat mot kroniska HCV infektioner.

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