Acta Universitatis Latviensis, 2007, Vol. 723, Biology, pp. 123–143

Is vaccine against HCV possible?

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Abstract

Natural immunity against the hepatitis C virus (HCV) was discovered not long ago. Some infected individuals are able to clear the virus, while other patients need intensive antiviral drug treatment that is expensive and not always effective. For this reason, many efforts are directed towards development of an effective vaccine in order to improve treatment outcome by stimulating humoral and cellular immune responses against HCV proteins or their immunodominant epitopes. Several HCV vaccine candidates have been tested in chimpanzees and promising results have allowed optimism about the development of at least partially effective vaccine against highly heterogeneous HCV pathogen. Hepatitis B virus core particles can be used successfully as a carrier of HCV epitopes. Chimeric HBc/HCV virus-like particles represent an interesting and valuable tool in the development of HCV vaccine.

Key words: hepatitis C virus, immune response, vaccine, virus-like particles.

Introduction

An estimated 3 % of the world's population (more than 170 million people) are infected by the hepatitis C virus (HCV). Most infections have become chronic: a condition that is incurable in many patients, leading to cirrhosis, end-stage liver disease and hepatocellular carcinoma (HCC). There were 1339 cases of chronic hepatitis C and 105 cases of acute hepatitis C in Latvia in the period from January 2006 to December 2006 (http://www.sva.lv/epidemiologija/statistika/). The number of chronic HCV patients increased by 30 % compared to year 2005, and by 76 % compared to statistical data acquired during the last five years (http://www.sva.lv/epidemiologija/statistika/). Current medical treatment options are limited. Chronic HCV infection is the most common cause of liver transplantation. Despite the discovery of the virus by molecular biological methods more than 15 years ago, and the sequencing of its entire genome, our knowledge of the virus and the nature of the protective immune responses is limited. Researchers have been hampered by the lack of a robust cell-culture system yielding infectious virus until very recently, and by the absence of a non-primate animal model. The situation clearly emphasizes the need for novel prophylactic/therapeutic approaches that can prevent spread of HCV and provide more efficient antiviral therapy of individuals suffering from chronic hepatitis C.

It is assumed that induction of vigorous, long-lasting, and cross-reactive antiviral antibodies as well as a multispecific cellular immune response that includes both helper and cytotoxic T lymphocytes (CTL) are necessary for an effective HCV vaccine (Rollier et al. 2004; Torresi et al. 2004; Neumann-Haefelin et al. 2005; Bowen, Walker 2005; Rehermann, Nascimbeni 2005). The development of such a vaccine meets, however, with many difficulties. The natural course of HCV infection and mechanisms of HCV interaction with an infected host are very complicated and still poorly understood, and the immunologic correlates associated with disease resolution and protection remains to be precisely defined. In addition, HCV is characterized by high genome sequence variability and a quasispecies distribution in an infected patient. The rare and expensive chimpanzee remains the only available animal model for HCV. Despite all these difficulties and restrictions some progress has been achieved during the last few years and several vaccine candidates are being explored. Among them are a recombinant protein subunit vaccine, a peptide vaccine, a live recombinant vaccine, virus-like particles (VLPs), and a DNA vaccine (Lechner et al. 2002; Inchauspe, Feinstone 2003; Duenas-Carrera 2004; Torresi et al. 2004; Houghton, Abrignani 2005; Encke et al. 2005). All these approaches are promising, although each of them has some shortcomings and limitations, and their applicability in medical practice remains to be determined. Most probably, the efficient HCV vaccine of the future will be based on a combination of two or several immunogens, one of which might be multi-epitope virus-like particles. One of the most promising VLP candidates is a chimeric particle on the basis of hepatitis B virus core antigen (HBcAg). The major intrinsic advantage of recombinant HBc particles is their improved immunogenicity due to formation of a covalent link between B and T helper (Th) epitopes, and the ability of HBcAg to act as both T-cell-dependent and independent antigen. As a result, the HBc particles induce high titers of antibodies and vigorous T-cell proliferative responses (Ulrich et al. 1998; Pumpens, Grens 2001). Perspectives of using HBc particles were demonstrated most recently in a successful clinical phase I trial of a malaria vaccine based on the HBcAg-platform (Oliveira et al. 2005).

Biology of the HCV virus

HCV is a noncytopathic hepatotropic member of the *Flaviviridae*, genus *Hepacivirus*, and is most closely related to the pestiviruses, Bovine viral diarrhea virus and GB virus B. All members of this family are small-sized, enveloped viruses containing a positive-strand RNA genome encoding a viral polyprotein. The viral genome of HCV is ~9.6 kilobase-long containing a single open reading frame (ORF). The ORF is flanked by 5' and 3' untranslated regions (UTRs). The highly conserved 5' UTR is 341-344 nucleotides long. Several stem-loop structures in this region contribute to an internal ribosome-binding site (IRES) that mediates the CAP-independent translation of the viral RNA. The ORF encodes a polyprotein of 3,010 or 3,011 amino acids, which is processed into structural and non-structural proteins (Fig. 1). The structural proteins forming the viral particle include the core protein and the envelope glycoproteins E1 and E2. The non-structural proteins include the p7 ion channel, the NS2-3 protease, the NS3 serine protease and RNA helicase, the NS4A polypeptide, the NS4B and NS5A proteins and the NS5B RNA-dependent RNA polymerase (Moradpour et al. 2007; Fig. 1). The HCV life cycle is entirely cytoplasmic. Replication occurs through a minus-strand intermediate in a membrane-

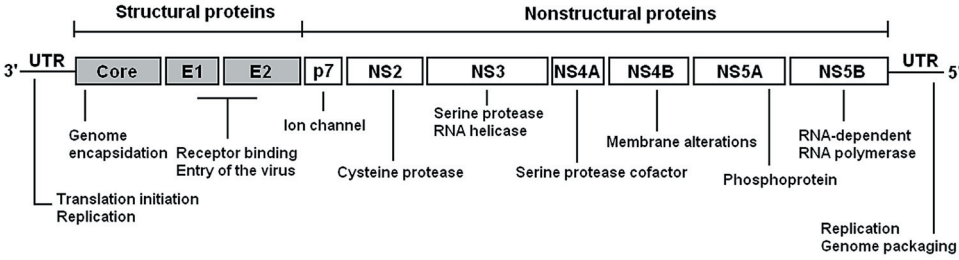


Fig. 1. HCV genes and gene products. The structure of the viral genome with the long open reading frame encoding structural and nonstructural proteins, and 5' and 3' untranslated regions (UTRs).

bounded compartment (Moradpour et al. 2004), yielding double-stranded RNA (dsRNA) intermediates. The replicative intermediates are fully exposed to the cell dsRNA-sensing machinery (Samuel 2001; Yoneyama et al. 2004) and induce strong innate cellular responses following infection. HCV infects only humans and chimpanzees.

HCV isolates can be classified into genotypes and subtypes (Simmonds et al. 2005). There are six major genotypes of HCV worldwide that differ by up to 30 - 35 % in sequence. Patients infected with genotype 1 do not respond as well to interferon-α-based therapy as those infected with genotype 2 or 3. Within an HCV genotype, several subtypes (designated a, b, c and so on) can be defined that differ in their nucleotide sequence by 20 - 25 % (Moradpour et al. 2007). The term quasispecies refers to the genetic heterogeneity of the population of HCV genomes that coexist in an infected individual (Simmonds et al. 2005).

Characterization of HCV infection

The hepatitis C virus causes acute and chronic hepatitis, and hepatocellular carcinoma (Hoofnagle 2002). The liver is its primary target organ, and the hepatocyte is its primary target cell. Acute infection is usually asymptomatic, making early diagnosis difficult. A notable feature of HCV infection is its tendency towards chronicity: ~70 % of acute infections become persistent, and chronic cases are often associated with serious liver disease (Hoofnagle 2002). As a result, HCV infection is a leading killer worldwide and the commonest cause of liver failure. In common with hepatitis B and human immunodeficiency (HIV) viruses, HCV is primarily transmitted parenterally (Alter 1996). Before the development of diagnostic tests, the infection was commonly passed on through blood and related products (Alter 1997), haemodialysis (Alter 1999) and organ transplantation (Alter 2002). Today, HCV primarily affects injecting drug users and their sexual partners (Alter 2002). It is a particular problem in correctional facilities, where 20 - 40 % of inmates are infected, in contrast to ~2 % of the general population (Spaulding 1999). It is opportunistic in HIV-infected individuals, ~25 % of whom are co-infected with HCV (this figure raises to 50 - 90 % among injecting drug users; Sulkowski 2000). Co-infection causes higher HCV titres and a more rapid progression to cirrhosis (Sulkowski 2000).

Immune response to HCV

In common with other persistent viruses, HCV does not kill the cells it infects, but triggers an immune-mediated inflammatory response (hepatitis) that either rapidly clears the infection or slowly destroys the liver, causing the development of HCC. The outcome is largely determined by the efficiency of the antiviral immune response. Host-virus interactions are ideally investigated in cell culture and small-animal models; the former are only now becoming available.

Implication of host determinants

Innate immune response. HCV spreads rapidly in the liver after inoculation (Rehermann, Nascimbeni 2005; Wieland and Chisari 2005), and thus the innate immune response might be expected to influence the outcome of infection. Indeed, prospective genomic analysis of the intrahepatic innate immune response in acutely infected chimpanzees suggests that HCV triggers a strong type-1 interferon (IFN-α/β) response as it spreads (Bigger et al. 2004; Wieland, Chisari 2005), but resists the effector functions of the downstream antiviral target genes that it induces. Importantly, the response is similar in animals that clear the infection and those that become persistently infected (Bigger et al. 2004; Wieland, Chisari 2005), implying that any influence on the outcome is indirect or obscure. Whatever its function, the innate intracellular immune response probably has a role in controlling HCV infection because the virus has developed several strategies to evade it. Several groups have shown an association between certain human leukocyte antigen (HLA) alleles and the outcome of HCV infection (Shoukry et al. 2004). These differences might influence the breadth of the T-cell receptor (TCR) repertoire and the ease with which the virus can escape. Confirmation of this hypothesis would be facilitated by an inbred mouse model of HCV infection.

Adaptive immune response. The clearest determinants of the outcome of HCV infection are the magnitude, diversity and quality of the adaptive immune response. Viral clearance during self-limited infection is characterized by vigorous polyclonal $CD4^+$ and $CD8^+$ T-cell responses that are relatively weak and narrowly focused in chronically infected humans and chimpanzees. Moreover, the onset of viral clearance and liver disease coincide with that of the T-cell response and the entry of virus-specific T cells into the liver; primary failure to induce a T-cell response or functional exhaustion of an initially vigorous response predict viral persistence (Shoukry et al. 2004; Rehermann, Nascimbeni 2005). However, the basis for variable immunological responsiveness to HCV has largely remained elusive. Indeed, it is not known whether the failure to respond vigorously in persistently infected subjects is caused by antigen overload during immunological priming, virus-induced defects in antigen presentation, hyperinduction of regulatory T cells, genetically determined restriction of the virus-specific T-cell repertoire or other causes (Bowen, Walker 2005). Therefore, whereas both primary and secondary immunological hyporesponsiveness to HCV seem to contribute to the establishment and maintenance of persistent infection, the reasons why they occur in selected subjects remain to be determined. Moreover, the virus can persist despite a multispecific $CD4^+$ and $CD8^+$ T-cell response (Shoukry et al. 2004; Rehermann, Nascimbeni 2005) by progressive mutational escape, which confirms the importance of the immune response in viral clearance and disease pathogenesis.

Implication of viral determinants

The six distinct genotypes of HCV show marked differences in geographic distribution, disease progression and response to therapy. However, the complex epidemiological differences in patient groups infected with each genotype make it difficult to ascribe variability in outcome to the virus instead of the host (Feld, Hoofnagle 2005). The mutation rate of HCV is high (10^{-3} per nucleotide per generation), as is its replication rate (~10^{12} virions per day in humans; Neumann et al. 1998). This results in explosive expansion of the virus after inoculation and in the evolution of numerous viral quasispecies in each infected subject, which could influence the magnitude and efficacy of the antiviral immune response. Moreover, the virus produces a constant stream of escape variants that outrun the immune response and can eventually produce mutants with no corresponding receptors in the immunological repertoire (Shoukry et al. 2004; Rehermann, Nascimbeni 2005). The influence of these parameters on the outcome of infection has been studied in a few acutely infected humans and chimpanzees and in many chronically infected individuals. The results show that B- and T-cell escape mutants are selected by the immune response during HCV infection and probably contribute to viral persistence. Regarding the second strategy, mutational inactivation of B- and T-cell epitopes is common in HCV infection (Bowen, Walker 2005). B-cell epitopes are concentrated in the hypervariable region 1 (HVR1) of the E2 protein (Mondelli et al. 2001), probably allowing the virus to persist in the presence of antibody that is neutralizing for its ancestors.

The T-cell epitope mutations span the viral polyprotein (Bowen, Walker 2005), often in residues that bind to major histocompatibility complex (MHC) molecules or are otherwise involved in antigen presentation. Mutations also occur in residues engaged by the TCR, making infected cells invisible to T cells expressing the corresponding TCR (Meyer-Olson et al. 2004). Although mutational escape probably contributes to the persistence of the virus, it is less clear whether it determines the outcome.

Development of vaccine against HCV

A decade ago, an effective vaccination against HCV was considered only a remote possibility. Three factors contributed to this: the high propensity of HCV to promote chronic persistent infections (Alter, Seeff 2000); evidence that convalescent humans and chimpanzees could be reinfected following re-exposure (Lai et al. 1994); and the considerable genetic heterogeneity of this positive-stranded RNA virus (Simmonds 2004). The situation today is more positive for two reasons. First, we now know that spontaneous eradication of the virus occurs in up to 30 % of acute infections (Seeff 2002) and that this viral clearance is associated with specific immune responses to the virus. Recapitulation of such immune responses by appropriate vaccination is therefore a realistic option. Second, clear evidence for at least some natural immunity has emerged recently in both humans (Mehta et al. 2002) and chimpanzees (Bassett et al. 2001; Weiner et al. 2001; Lanford et al. 2004). Chimpanzees are the only animal model available and develop only mild clinical sequelae. Convalescent humans and chimpanzees are protected against re-exposure to the virus in the majority of cases, even against very divergent viral strains. Importantly, protection is usually at the level of prevention of progression to chronic, persistent infection following re-exposure rather than prevention of acute reinfection, but this could translate to effective prophylaxis because, in humans, it is the chronic, persistent nature

of HCV infection that is mainly associated with viral pathogenicity (Alter, Seeff 2000; Seeff 2002). Although some re-exposed individuals develop chronic infection (Farci et al. 1992), most do not (Bassett et al. 2001; Weiner et al. 2001; Mehta et al. 2002; Lanford et al. 2004). This suggests that the generation of at least a partly effective vaccine against HCV is feasible. Indeed, emerging vaccine efficacy data from the chimpanzee challenge model indicate that it is possible to impede the progression to chronic infection in vaccinees. Until very recently (Lindenbach et al. 2005; Wakita et al. 2005; Zhong et al. 2005), it was not possible to grow HCV efficiently in cell culture, and thus the use of inactivated or live attenuated viral vaccines has not yet been evaluated. Vaccine approaches have therefore included the use of adjuvanted recombinant polypeptide subunits of the virus in attempts to prime viral neutralizing antibodies to the envelope glycoproteins 1 and 2 (gpE1 and gpE2), as well as priming MHC class-II-restricted $CD4^+$ Th and MHC class-I-restricted $CD8^+$ CTL responses to these and other viral proteins. Both types of T cell can secrete antiviral cytokines such as interferon-α (IFN-α), and $CD8^+$ CTLs have the potential to kill infected cells.

Results from the recent studies have shown optimistic observation of successfully vaccination against HCV. These studies involved the use of the recombinant HCV envelope glycoproteins gpE1 and gpE2 as vaccine antigens. Derived from mammalian cells, the two glycoproteins associate together to from a non-disulphide linked gpE1-gpE2 heterodimer that is thought to resemble the pre-virion envelope structure (Ralston et al. 1993). When combined with oil/water-based adjuvants and used to vaccinate naive chimpanzees, this vaccine candidate elicits anti-envelope antibodies as well as Th cell responses to gpE1 and gpE2. Some earlier experiments showed that when vaccinated animals were challenged experimentally with homologous viral inocula, the highest responding animals (in terms of anti-gpE1/gpE2 antibody titres) were completely protected against infection (Choo et al. 1994). Using sensitive reverse transcription polymerase chain reaction (RT-PCR) assays, no viraemia was detected in blood or liver samples at any time after challenge in these seemingly 'sterilized' animals. This apparent sterilizing immunity correlated directly with anti-gpE2 antibody titres that prevent the binding of gpE2 (or the virus itself) to CD81 (Rosa et al. 1996), which has been shown to be an important receptor component for binding of infectious HCV (Pileri et al. 1998; Lindenbach et al. 2005; Wakita et al. 2005; Zhong et al. 2005) and for cell entry of lentiviral/HCV pseudoparticles (McKeating et al. 2004). Furthermore, although lower-responding animals became infected, the majority underwent an abortive acute infection that did not result in the persistently infected carrier state (Choo et al. 1994) that in humans can be associated with chronic liver disease (Alter, Seeff 2000; Seeff 2002). Overall, these data showed that the carrier rate in vaccinees was significantly lower than in unimmunized controls (Choo et al. 1994; Houghton, Abrignani 2005). A crucial question that remained for the authors to solve was whether the vaccine derived from strain HCV-1 would protect against heterologous strains of the virus. For that purpose they challenged nine chimpanzee vaccinees with the HCV-H strain that, like the vaccine strain HCV-1, was of the 1a genotype. Although none of the vaccinated animals was protected against acute infection, all but one vaccinee resolved the acute infection and failed to progress to the carrier state (Houghton, Abrignani 2005). By contrast, the majority of control animals became carriers when challenged with HCV-H, indicating that the vaccine significantly reduced chronic, persistent infection (Houghton, Abrignani 2005). These pre-clinical data [and supporting data from other small studies

exploring various gpE1/gpE2 vaccine formulations (Forns et al. 2000; Puig et al. 2004; Rollier et al. 2004)] supported the authors to initiate a clinical prophylactic programme using adjuvanted gpE1/gpE2 that is currently in phase 1 testing.

It is difficult to prime $CD8^+$ CTLs using polypeptide subunit vaccines, although certain adjuvants are capable of eliciting such responses (Polakos et al. 2001; Pearse, Drane 2005). Various forms of plasmid DNA vaccine (Table 1) are also being explored to elicit HCV-specific humoral and cellular immune responses to encoded antigens which, by virtue of being newly synthesized in the cytosol of transfected cells, can be particularly effective at priming $CD8^+$ CTLs. DNA vaccines also include immunostimulatory deoxycytosine-deoxyguanosine (CpG)-containing motifs capable of activating antigen-presenting dendritic cells (Houghton, Abrignani 2005). This would lead to stimulation of innate immune responses (such as the synthesis of type 1 interferons and natural killer (NK) cells) as well as adaptive B- and T-cell responses to vaccine antigens. Various live attenuated

Table 1. Prophylactic HCV vaccine candidates

Vaccine	Potency	Stages
Recombinant gpE1/gpE2 in oil/water adjuvants (Choo et al. 1994; Houghton, Abrignani 2005)	Protects chimpanzees against chronic infection	Phase 1 clinical trials
DNA prime and protein boost (using C, gpE1, gpE2 and NS3) (Rollier et al. 2004)	Protection or amelioration in chimpanzee challenge model	Pre-clinical
Recombinant VLPs containing C, gpE1, gpE2 (Jeong et al. 2004)	Highly immunogenic in mice and baboons	Pre-clinical
Recombinant gpE1 in alum (Leroux-Roels et al. 2004)	Primes humoral and cellular immune responses in humans	Phase 1/2 clinical trials
Modified vaccinia ankara expressing gpE1/gpE2 (Abraham et al. 2004)	Induces Th1 response in HLA A2.1 mice	Pre-clinical
Semliki forest virus expressing NS3 (Brinster et al. 2002)	Induces NS3-specific CTLs in mice	Pre-clinical
DNA encoding gpE1/gpE2 in poly-lactide-co-glycolide particles (O'Hagan et al. 2004)	Substantial increase in anti-gpE1/E2 titre in mice compared with naked DNA	Pre-clinical
Defective ovine atadenovirus expressing NS3 (Wuest et al. 2004)	Strong Th1 cellular response in mice	Pre-clinical
DNA prime and canary pox boost (encoding all HCV genes) (Pancholi et al. 2003)	Broad Th1 cellular immune responses in mice	Pre-clinical
Defective alphaviral particles expressing gpE1/gpE2 and NS genes (Perri et al. 2003)	Mouse studies in progress	Pre-clinical
Recombinant NS3 protein with polyriboinosinic: polyribocytidylic acid emulsified in Montanide ISA 720 (Jin et al. 2007)	Strong and persistent Th1 cellular immune responses in mice	Pre-clinical
Recombinant core protein (aa 1-122) formulated in Montanide ISA 720 with CpG oligodeoxynucleotides (Roohvand et al. 2007)	Strong humoral and cellular immune response in mice	Pre-clinical

or defective viral or bacterial vectors expressing HCV genes (Table 1) are also being investigated because improved vaccine immunogenicity can result from more efficient expression and delivery of HCV antigens. This may include the targeting of antigen-presenting cells in some cases. The use of various prime/boost immunization modes and regimens (Table 1) are also being explored to optimize vaccine immunogenicity and potency (Houghton, Abrignani 2005).

Potential for therapeutic HCV vaccination

The current standard-of-care therapy for chronically infected HCV patients is a combination of pegylated IFN-α and ribavirin, which is costly, lengthy (6 to 12 months), associated with significant side effects and results in sustained viral response in only ~50 % of patients. In patients infected with genotype 1 response rates are even lower (Saadeh, Davis 2004). With an estimated 170 million HCV carriers worldwide, it is clearly important to develop better therapeutic options. With our increasing knowledge of the virus-encoded enzymes and genetic elements vital to the life-cycle of HCV, much attention is now being focused on the development of HCV protease, replicase, helicase, antisense, silencing RNA and other specific inhibitors. However, preliminary data have directly linked responses to IFN-α and ribavirin with pretreatment titres of viral antibodies (Baumert et al. 2000; presumed to be against the envelope glycoproteins), peripheral Th cell responses to the HCV core and other antigens (Cramp et al. 2000), as well as to intrahepatic $CD8^+$ CTL responses to the virus (Nelson et al. 1998). Total pretreatment $CD8^+$ T-cell counts in the liver have also been correlated with sustained responses to standard-of-care therapy (Vrolijk et al. 2003). Therefore, it may be possible to boost such immune responses in patients by appropriate vaccination and thereby improve the response rate to the standard-of-care therapy. Such immunotherapy may also help control the emergence of escape mutants that would be predicted to arise from any future use of HCV protease or replicase inhibitors, for example, given the extreme fluidity and heterogeneity of the HCV genome (Simmonds 2004). Many therapeutic vaccine trials are planned or are already in progress and use diverse delivery methods and formulations (summarized in Table 2) but little information is available about their efficacy at present. What is known, however, is that use of an alum-adjuvanted recombinant gpE1 antigen was able to boost humoral and cellular immune responses to gpE1 in viraemic patients, providing encouragement that vaccination can increase immune responses in pre-existing carriers (Nevens et al. 2003). It remains to be seen whether boosting viral-neutralizing antibody titres or broad $CD4^+$ Th responses or broad $CD8^+$ T-cell responses will have the greatest impact on reducing viral load and in the response to antiviral therapy. But, as may be the case for optimal prophylaxis, boosting all of these immune responses may be ideal for immunotherapy. HCV tries to counter innate immunity by inhibiting the induction of type-1 interferons (Foy et al. 2005; Li et al. 2005) and downregulating NK cell activity (Crotta et al. 2002; Tseng, Klimpel 2002).

Therefore, therapeutic vaccine formulations could benefit by inclusion of molecules capable of triggering innate immune responses. Such molecules include oligonucleotides containing CpG motifs that trigger Toll-like receptor 9 within dendritic cells and that also enhance adaptive immune responses to vaccine antigens (Abel et al. 2005). If successful, vaccination for the treatment of chronic hepatitis C would be one of the first demonstrations of immunotherapeutic intervention in chronic viral infections, although,

Table 2. HCV immunotherapeutic vaccine candidates

Vaccine	Potency	Stage
Alum-adjuvanted E1 glycoprotein (Nevens et al. 2003)	Boosts humoral and cellular immune responses to gpE1 in HCV patients. May ameliorate hepatitis	Phase 1/2 patient trials
Oil/water-adjuvanted gpE1/gpE2 proteins (Choo et al. 1994; Houghton, Abrignani 2005)	Prophylactic efficacy in chimpanzees. Boosts anti-gpE1/gpE2 antibody titres in chronically infected HCV chimpanzees	Phase 1b patient trials
ISCOMATRIX-adjuvanted (Polakos et al. 2001; Pearse et al. 2005) core protein	Primes Th1-type $CD4^+$ and $CD8^+$ CTL responses in macaques and uninfected humans to conserved epitopes within core antigen	Phase 1b patient trials
ISCOMATRIX-adjuvanted (Polakos et al. 2001; Pearse et al. 2005) NS3-NS4-NS5-C polyprotein	Primes broad Th1-type $CD4^+$ and $CD8^+$ CTL responses in chimpanzees which when challenged with heterologous HCV have reduced viraemia and hepatitis relative to control	Pre-clinical
Heat-killed yeast expressing C and NS3 (Franzusoff et al. 2005)	Primes specific $CD4^+$ and $CD8^+$ T cells in mice	Pre-clinical

very recently, such an approach has been used successfully to inhibit the age-related emergence of herpes zoster infections and disease in carriers (Oxman et al. 2005).

Hepatitis B virus core for display of foreign epitopes

Hepatitis B virus (HBV) core protein or antigen (HBcAg) was first reported as a promising VLP carrier in 1986 (Newton 1987) and published in 1987 (Clarke et al. 1987). The HBcAg particles were the beginning of a long list of structurally well-defined icosahedral VLP carriers and to this day remain one of the most flexible and immunologically most powerful epitope carrier candidates. HBcAg consists of 180 or 240 copies of identical polypeptide subunits. The multifunctional character of HBcAg seems to be responsible for the unusual flexibility of the core protein. The HBc polypeptide is able to self-assemble and was therefore selected as a target for protein engineering manipulations.

In many ways HBcAg holds a unique position among other VLP carriers because of its high expression level and efficient particle formation in mostly all known homologous and heterologous expression systems, including bacteria. Over-expressed HBc protein showed correct self-assembly into naturally-shaped particles in the absence of any other viral component. Electron microscopy revealed the ultrastructural identity of the HBc particles derived either from HBV virions and infected hepatocytes, or from *Escherichia coli* (Cohen, Richmond 1982) or yeast (Yamaguchi et al. 1988).

The fine structure of HBc particles (Fig. 2) was revealed by electron cryomicroscopy and image reconstruction (Crowther et al. 1994; Bottcher et al. 1997; Conway et al. 1997). Finally, this three-dimensional structure was confirmed by X-ray crystallography at 3.3-Å

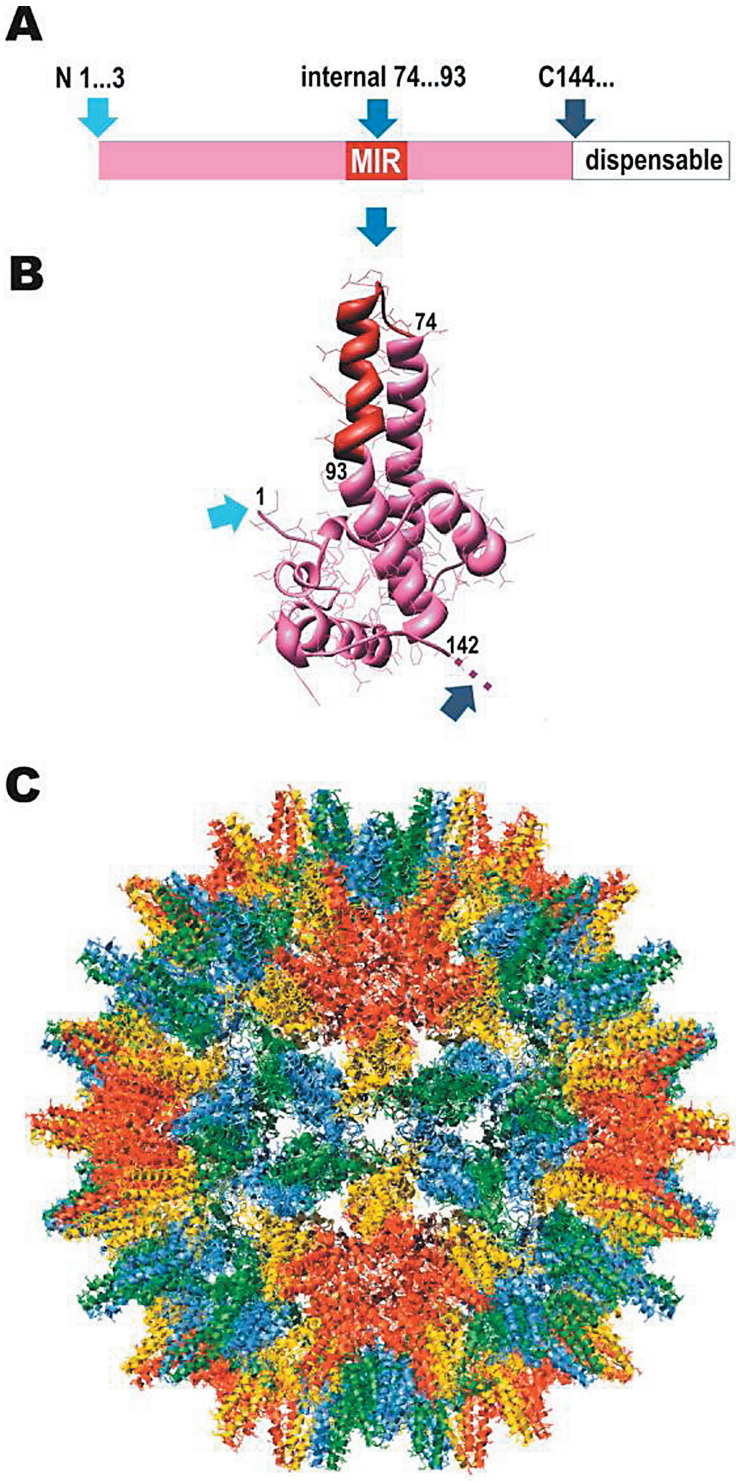
resolution (Wynne et al. 1982). Organization of HBc particles was found largely α-helical (Fig. 2B, C) and quite different from previously known viral capsid proteins with β-sheet jelly-roll packings (Wynne et al. 1982; Crowther et al. 1994). The HBc monomer fold is stabilized by a hydrophobic core that is highly conserved among human viral variants. Association of two amphipathic α-helical hairpins results in the formation of a dimer with a four-helix bundle as the major central feature. The dimers are able to assemble into two types of particles, large and small, that are 34 and 30 nm in diameter and correspond to triangulation number T = 4 and T = 3 packings, containing 240 and 180 HBc molecules, respectively. The major immunodominant region (MIR) with the central positions amino acids (aa) 76-81 is located at the tips of the α-helical hairpins (Fig. 2B) that form spikes on the capsid surface (Wynne et al. 1982).

In addition to MIR, the region aa 127-133 is the next exposed and accessible epitope on the particle surface. This region is located at the end of the C-terminal α-helix and forms small protrusions on the HBcAg surface.

Of special structural value was the clear demonstration of dispensability of the C-terminal protamine-like arginine-rich domain of the HBc protein (aa 150-183) for its self-assembly capabilities in the so-called HBcΔ particles (Borisova et al. 1988; Gallina et al. 1989; Inada et al. 1989). The HBcΔ particles formed by C-terminally truncated polypeptides were almost indistinguishable from the HBc particles formed by full-length HBc polypeptides, as shown by electron cryomicroscopy (Crowther et al. 1994). However, unlike the full-length HBc particles, HBcΔ particles were less stable, failed to encapsidate nucleic acid, and usually accumulated as empty shells (Borisova et al. 1988; Birnbaum, Nassal 1990; Bundule et al. 1990; Hatton et al. 1992; Ulrich et al. 1992; Crowther et al. 1994). The C-terminal limit for self-assembly of HBcΔ particles was mapped experimentally between aa residues 139 and 144 (Inada et al. 1989; Birnbaum, Nassal 1990; Seifer, Standring 1995).

The extremely high immunogenicity of HBcAg particles has been known for a long time. Thus, HBV patients develop a strong and long-lasting humoral anti-HBc response (Hoofnagle et al. 1973). Among the HBV polypeptides, HBc induces the strongest B-cell, T-cell, and CTL response (Chisari, Ferrari 1995). HBcAg is known to function as both a T-cell-dependent and T-cell-independent antigen (Milich, McLachlan 1986). Following immunization, it primes preferentially Th1 cells, does not require an adjuvant (Milich et al. 1997a), and is able to mediate anti-HBs response (Milich et al. 1987). Recently, enhanced immunogenicity of HBcAg was explained by its ability to be presented by B cells as the primary antigen to T cells in mice (Milich et al. 1997b). HBcAg elicits a strong CTL response

Fig. 2. General structural features of the HBc protein as a putative VLP carrier. A linear presentation of the HBc gene with localization of the (i) MIR (major immunodominant region), (ii) self-assembly dispensable protamine-like C-terminal region (aa 145-183), and (iii) preferable sites for the insertion of foreign epitopes (shown by blue arrows) (A). A three-dimensional presentation of the HBc monomer (chain A) derived from the crystal structure (Wynne et al. 1999) with localization of alpha-helices and MIR (colored red), insertion sites for foreign epitopes are marked by the arrows as in the (A). The N- and C-terminal, as well as MIR border amino acid residues of the map are deciphered (B). The T = 4 HBc capsid viewed down an icosahedral threefold axis (C). HBc chains are colored as follows: A - orange red, B - gold, C - green, D - blue. The maps are the generous gift of R. Anthony Crowther. Molecular graphics images were produced using the UCSF Chimera package (Pettersen et al. 2004) from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081).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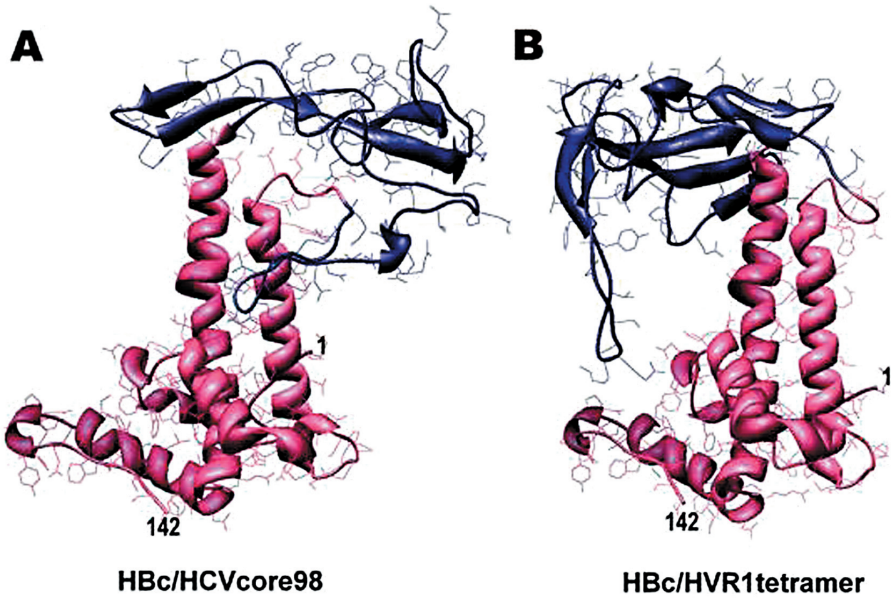

Fig. 3. Prediction of the spatial structure of the chimeric HBc/HCV monomers with insertions at MIR: HBcMIR/HCVcore98 (A) and HBcMIR/HVR1tetramer (B). An X-ray based three-dimensional map of the molecules was calculated on the basis of the HBc carrier X-ray data (T = 4, resolution 3.3 Å; Wynne et al. 1999) by the 3D-JIGSAW program (Contreras-Moreira, Bates 2002) and presented by the Chimera program (see Fig. 2). HCV inserted sequences are colored blue. The N- and C-terminal amino acid residues of the maps are deciphered.

during HBV infection (Mondelli et al. 1982), and this response is maintained for decades following clinical recovery, apparently keeping the virus under control (Rehermann et al. 1996).

Experimental search for appropriate target sites for foreign insertions pointed to the MIR region at the tip of the spike and to the N and C termini of the HBc molecule (Milich et al. 1995; Pumpens et al. 1995; Schodel et al. 1996; Ulrich et al. 1998; Murray, Shiau 1999; Pumpens, Grens 1999; Karpenko et al. 2000; Pumpens, Grens 2001). These findings are in general agreement with the X-ray data because these regions do not participate in the critical intra- and intermolecular interactions (Wynne et al. 1999).

HBc/HCV chimeric proteins as a model for an HCV vaccine

Several experiments on insertion of HCV protein fragments into HBc vectors were already done, but the purpose of these experiments was different. C-terminal insertions of the HCV core protein demonstrated the extraordinary capacity of the HBc particle as a VLP carrier: a 559-aa-long insertion did not prevent self-assembly of chimeras, and even a 741-aa-long insertion allowed production and self-assembly of chimeras to some extent (Yoshikawa et al. 1993). C-terminally added HCV core (Wu et al. 1999) and NS3 (Claeys et al. 1995) sequences were used successfully for detection of specific antibodies in HCV enzyme immunoassay.

Important practical advantage of the HBc model lies in the fact that chimeric HBc-derived particles due to their particulate nature are easy to purify by gel filtration or sucrose gradient centrifugation (Pumpens, Grens 2001). C-terminally truncated variants can be subjected to dissociation with subsequent re-association, in order to remove internal impurities and produce nucleic acid-free preparations. A special purification protocol for preparation of HBc derivatives of vaccine quality was elaborated by addition of a 6 histidine tag to the truncated C-terminus of the HBc protein (Wizemann, von Brunn 1999). On the other hand, the ability of full-length or special chimeric HBc derivatives to controlled encapsidation of nucleic acids may be used for the further development of this carrier for gene therapy experiments (Pumpens, Grens 2001).

One of the crucial questions in construction of chimeric VLPs is whether they will retain the ability of formation of complete, well-structured and stable capsids after insertion of foreign sequences. There are some modeling programs which allow prediction to some extend or at least visualization how chimeras could look like. But nevertheless computer modeling is not able to provide an answer to this important problem, and the only path is to try everything in practice. For the construction of HBc/HCV immunogen we have chosen two HCV determinants: HCV core aa 1-98 and HCV HVR1 region. HCV core 1-98 and HVR1 insert (created of four HVR1 variants; HVR1tetramer) were inserted at MIR as well as fused to C terminus of truncated HBc. Detailed information on description of chimeric HBc/HCV VLP construction, purification and analysis was published by Mihailova et al. 2006a. Chimeric proteins with insertions at MIR (HBcMIR/HCVcore98 and HBcMIR/HVR1tetramer) failed to form VLPs in both cases. Based on the predictions made by the 3D-JIGSAW program (Contreras-Moreira, Bates 2002) for these two chimeric constructs (Fig. 3), foreign inserts made of predominantly β-sheets do not form compact structure on the tip of the spike of the monomer of HBc protein but somewhat flat formation. Considering that basic unit of HBc VLPs is the dimer of HBc polipeptides such flat formation could be the reason for disruption of VLP organization. Indeed, electron microscopy analysis confirmed that HCV core 1-98 and HVR1tetramer inserts at MIR of HBc formed only clumps of uncompleted or disrupted capsids (Fig.4 B, D). Our experiments show that computer modeling can be successfully used for prediction of VLP formation; although these data should be confirmed experimentally. Two other constructs containing inserts at C terminal part of truncated HBc (HBcCterm/HCVcore98 and HBcCterm/HVR1tetramer) formed complete virus-like particles (Fig.4 C, E). Although construct with HCV core insert at C terminus failed to induce strong HCV specific immune response in mice, the other construct bearing HVR1 multi-epitope showed very promising results. In the study we used not a single HCV HVR1 sequence but a combination of four HVR1 variants in order to construct a model of a multi-epitope immunogen capable of inducing antibodies reacting with a large number of HVR1 sequence variants. As a result, the sera of mice immunized with the chimeric HBc VLP bearing such a tetrameric molecule were able to react with 55 % of variants of HVR1 peptides derived from 172 field HCV isolates of different genotypes (Mihailova et al., unpublished data). The same sera were also able to inhibit the binding of HCV-like particles (comprised of E1/E2 proteins, derived using baculovirus expression system in insect cells) of different HCV subtypes (1a and 1b) to Huh7 cells in surrogate "neutralization" assay (Mihailova et al., unpublished data). HCV HVR1 tetramer as a Histidine tagged protein also showed very interesting results. The method describing HVR1tetramer construct, protein purification and immunization of

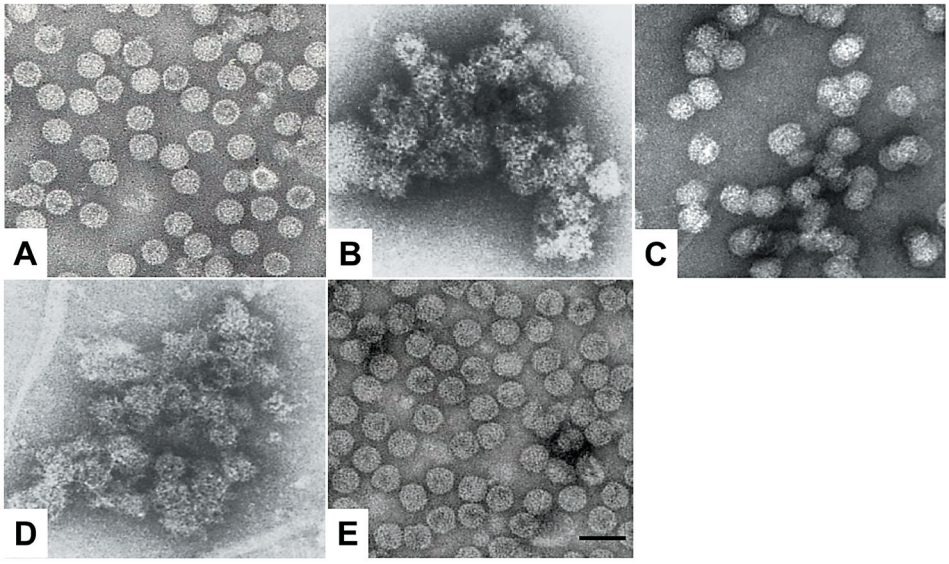

Fig. 4. Comparison of electron microphotographs of purified chimeric HBc/HCV particles with insertions at MIR or C terminus of HBc protein. HBc particles made of original truncated HBc protein (A) and of chimeric HBc/HCV proteins: HBcMIR/HCVcore98 (B), HBcCterm/HCVcore98 (C), HBcMIR/HVR1 tetramer (D), and HBcCterm/HVR1tetramer (E). Bar = 50 nm.

mice is available in a paper published by Mihailova et al. (2006b). The HVR1 tetramer complex was reactive with 75 % of chronically infected patients' sera including sera from patients infected with HCV subtypes 1a, 1d, 2b, 3a and 4. At the same time, the single component of our tetrameric HVR1 construct, the mimotope R9, alone was recognized only by 45 % of the sera and showed much more narrow subtype recognition (Mihailova et al. 2006b). Our finding indicates that it is possible to induce relatively broad and cross-reactive immune response against HCV.

Future directions

In the future, it will be important to use the chimpanzee model to further define correlates of protection, duration of vaccine-mediated protection, the extent of cross-protection against diverse genotypes and mechanisms of chronicity and to determine optimal vaccine formulations for prophylactic and immunotherapeutic efficacy. In addition, human cohorts at high risk of infection need to be identified and characterized for efficacy trials. The huge burden of chronically infected HCV patients facilitates the testing of various immunotherapeutic vaccine formulations that, most probably, will be especially useful when used as adjunct therapy with antiviral drugs, including pegylated IFN-α and ribavirin as well as the new class of HCV drugs currently under development that inhibit viral enzymes and other elements crucial to the viral life-cycle. It will also be important to understand the mechanisms involved in immune dysfunction and evasion during chronic HCV infections so as to facilitate the design of further immunotherapies.

Acknowledgements

We are grateful to Mr. J. Ozols (Riga, Latvia), Mrs. L.Kovalevska (Riga, Latvia) and Mrs. V. Ose (Riga, Latvia) for their help in the development of the VLP purification and analysis procedures, Dr. K. Uhde-Holzen (Aachen, Germany) for kind advice and discussion, Dr. M. Fiedler (Essen, Germany), Mr. C. Oniangue Ndza (Essen, Germany), Dr. G. Zelinskyy (Essen, Germany) and Dr. D. Skrastina (Riga, Latvia) for their kind help in work with mice. We would like to thank Prof. M. Roggendorf, the head of Institute of Virology in Essen, for the possibility to fruitful collaboration, for his help and advice. We also are thankful to Mr. Y. Khudyakov (Atlanta, USA) for the analysis of sera from immunized animals with synthetic peptide library. The work was supported by The European Social Fund (ESF) and Grant No. 05.1626 from the Latvian Council of Science.

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Vai vakcīna pret HCV ir iespējama?

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Kopsavilkums

Pavisam nesen tika atklāti fakti par iedzimto imunitāti pret hepatīta C vīrusu (HCV). Daži inficēti indivīdi tiek galā ar vīrusu, turpretī citiem pacientiem ir nepieciešama intensīva ārstēšana ar pretvīrusu preparātiem, kas ir dārga un ne vienmēr efektīva. Tādēļ, daudz pūļu velta tādas efektīvas vakcīnas izveidošanai, kas stimulētu humorālu un šūnu imūno atbildi pret HCV proteīniem un to imundominantiem epitopiem un ļautu uzlabot infekcijas iznākumu. Vairāki HCV vakcīnu kandidāti pārbaudīti šimpanzēs, un iegūtie rezultāti ļauj optimistiski skatīties uz vismaz daļēji efektīvas vakcīnas izstrādi pret tādu augsti heterogēnu patogēnu kā HCV. Hepatīta B vīrusa kora daļiņas var veiksmīgi izmantot kā HCV epitopu nesējas. Himerās HBc/HCV vīrusveidīgās daļiņas ir interesants un cerīgs līdzeklis HCV vakcīnu izveidē.