

Review

# The challenge of developing a vaccine against hepatitis C virus

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## 1. Introduction

Hepatitis C virus (HCV) is a single stranded positive-sense RNA virus belonging to the *Flaviviridae* family [1]. Members of this family are small enveloped viruses that have been classified into three different genera: *Pestivirus*, which contains animal pathogens such as bovine viral diarrhoea virus and hog cholera virus; *Flavivirus*, which contains mostly arthropod-transmitted human pathogens such as dengue fever and yellow fever viruses; and *Hepacivirus*, whose only member is HCV [1]. The recently discovered GB virus-B (GBV-B), which causes hepatitis in experimentally infected tamarins, will probably be classified with HCV [2,3].

HCV is an important human pathogen, but the scope of its impact on human health has only recently been truly appreciated. The prevalence of HCV infection in the general population varies depending on the geographical area and ranges from less than 1% in Northern Europe to as high as 20% in some developing countries such as Egypt. It has been estimated that approximately 170 million people are chronically infected with HCV worldwide [4]. However, despite effective screening of blood and blood products, and use of sterile techniques, acute HCV is still a problem in industrialized countries. For example, around 40 000 new HCV infections occur each year in the US [5] and the majority of individuals with acute infection become persistently infected [6]. The source of these infections is principally the illicit use of parenteral drugs. Exposure to contaminated blood, especially via contaminated needles, syringes and surgical instruments, also accounts for the spread of HCV in developing countries. Chronic HCV infection is an important cause of liver cirrhosis and hepatocellular carcinoma in the Western World and Japan and, furthermore, represents the most frequent indication for liver transplantation in developed countries.

Treatment of chronic HCV infection has improved considerably during the last few years: the combination of interferon and the nucleoside analogue ribavirin achieves a sustained virological response in approximately 40% of patients with chronic hepatitis C [7–9]. It is possible that during the next few years, new antiviral agents such as inhibitors of the viral protease, helicase or polymerase will further improve the response rate of the current therapeutic agents. However, antiviral therapy is not affordable in most developing countries, where the prevalence of HCV is generally the highest. Thus, given the huge reservoir of HCV worldwide, the development of an effective vaccine will be the only way to control disease associated with HCV infection.

## 2. The prospect of success for the development of HCV vaccines

Prophylactic vaccines against some other members of the *Flaviviridae* family already exist or are under development [1]. For example, a live-attenuated vaccine against yellow fever virus is available and has proven to be highly effective. Efforts have been made since the 1940s to produce dengue vaccines. Immunity acquired from natural infection is specific for each dengue serotype and infection of an individual with three different serotypes has been reported. For this reason, tetravalent vaccines are being developed. However, the development of an effective vaccine against HCV will probably be more difficult. Viruses such as dengue or yellow fever cause acute self-limited infections in humans, which means that the host immune responses are capable of controlling the disease, and also provide immunity against subsequent exposure. Despite the generation of humoral and cellular immune responses against HCV, only 15–30% of the individuals infected with HCV resolve their infection [10]. Additionally, it has been reported that HCV can cause more than one episode of acute hepatitis in the same individual [11]. Similarly, in the chimpanzee model, animals re-challenged with the same strain or with a closely

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related HCV strain were not protected against reinfection following acute resolving infection [12–14]. Thus infection with HCV apparently does not provide complete protection against subsequent exposures.

### 2.1. The genetic heterogeneity of HCV contributes to its ability to elude preexisting immunity

One of the possible explanations for the lack of protective immunity after HCV infection is the genetic heterogeneity of HCV [15,16]. HCV can be classified into six major genotypes and over 100 subtypes. The polyprotein sequences of the most diverse isolates differ by approximately 30% and their envelope proteins (E1 and E2) differ by up to 50% [15]. Within infected individuals, HCV circulates as a quasispecies, which is a mixture of closely related but distinct genomes [17]. Genetic variability is particularly high at the amino terminus of E2, the hypervariable region 1 (HVR1). Genetic evolution of the virus might permit HCV to escape the host immune surveillance. In fact, genomic changes in the virus can emerge in a pattern consistent with repeated selection by escape from the host humoral or cellular immune responses [18–21]. Farci et al. [22] demonstrated that antibodies raised against the dominant HVR1 sequence of an HCV strain could neutralize *in vitro* that strain but not minor species present in the inoculum when tested in the chimpanzee model (Fig. 1). Also, it has been observed that even a chimpanzee with effective immunity against homologous genotype 1a challenge was not protected against challenge with isolates of genotype 1b or 2a [23]. Therefore, the possibility that immunity against HCV is strain- or isolate-specific will have to be taken into consideration when developing an HCV vaccine. In addition,

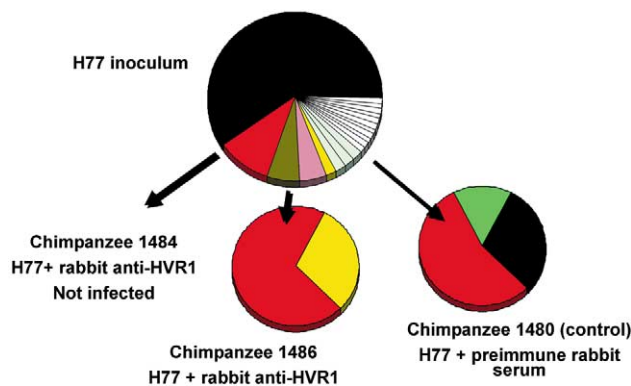
the apparent inherent ability of HCV to escape even specific immunity by developing escape mutants could result in vaccine failure as was recently described for simian immunodeficiency virus [24].

### 2.2. The reason for the high chronicity rate of HCV is poorly understood

HCV infection becomes chronic in most cases. However, the mechanisms by which HCV escapes the host immune responses and establishes a chronic infection are not well defined, but clearly a better understanding of the components involved would be valuable in designing vaccine candidates. As stated above, the development of immune escape mutants has been postulated as one of the main mechanisms of HCV persistence [25,26]. However, this does not appear to be the only mechanism involved in the establishment of a persistent infection. Differing quality of the host responses are most likely involved.

The recent development of infectious complementary DNA (cDNA) clones of HCV [27–30] has permitted more controlled studies of the role of viral evolution in the persistence of HCV in chimpanzees. Since the RNA that is used for transfection is generated from a single HCV sequence, the initial infection in the chimpanzee is monoclonal. Interestingly, despite the absence of a quasispecies during the early acute phase of the infection the majority of transfected animals developed a chronic infection [25]. Also the virus could persist without evolution in the envelope proteins, indicating that escape from neutralizing antibodies did not play a role, whereas mutations in the non-structural proteins could have represented cytotoxic T lymphocyte escape mutants. Furthermore, of two chimpanzees infected with recombinant HCV lacking the HVR1, one became chronically infected and the other cleared the infection, demonstrating that the most variable region of HCV was not the determinant of clearance of infection or progression to chronicity [31]. Although these experiments provide extremely important information, they do not reproduce a natural infection, where multiple viral strains (quasispecies) infect the host. In fact, the potential relevance of the quasispecies evolution in the outcome of HCV infection was recently demonstrated in a study of patients with acute hepatitis C following blood transfusions [32], which showed that an increase in viral population diversity during the acute phase of HCV infection was associated with evolution to chronicity.

As relates to the quality of the host responses, quantitatively inadequate cellular immunity might permit HCV persistence and contribute to the development of chronic liver disease [33,34] (see below). It is also possible that the virus itself might contribute to diversion or inhibition of the host immune responses to viral antigens by employing decoy antigens [35] or by inhibition of the host's interferon-inducible antiviral response [36,37].



**Fig. 1.** Genetic heterogeneity of HCV contributes to escape from preexisting immunity. Antibodies against the dominant sequence of the HVR1 of strain H77 were raised in a rabbit by repeated peptide immunization. The rabbit hyperimmune serum was mixed with the H77 inoculum and, after incubation, the mixture was inoculated into chimpanzees. One animal (chimpanzee 1484) did not become infected whereas a second animal (chimpanzee 1486) became infected. Sequence analysis demonstrated that a minor species present in the original inoculum was the predominant HCV strain in chimpanzee 1486, but the dominant sequence from the inoculum was not recovered from this chimpanzee [22].

### 2.3. Limited understanding of the immunologic correlates of HCV clearance

The existence of neutralizing antibodies has been shown in several experiments performed in chimpanzees. Farci et al. [38] demonstrated that serum from a patient chronically infected with HCV was able to neutralize HCV strains present in this patient 2 years before. Yu et al. [39] reported that immune globulin pools containing antibodies against the envelope HCV proteins and generated from approximately 200 anti-HCV positive blood donors could neutralize HCV: a chimpanzee inoculated with a mixture of anti-HCV positive immune globulin and virus did not become infected, whereas a control animal inoculated only with the virus developed HCV infection. Krawczynski et al. [40] investigated the utility of post-exposure prophylaxis in chimpanzees, using an immune globulin preparation made from plasma units collected from 460 anti-HCV positive patients. The treated chimpanzees had early clearance of viremia and did not develop acute hepatitis. A study performed in patients undergoing liver transplantation for HCV- and HBV-related liver cirrhosis showed that infusion of anti-HBs hyperimmune globulin manufactured before 1990 was associated with a low incidence of HCV infection of the graft [41]. These data strongly suggest that immune globulin produced before 1990 and containing anti-HCV was capable of neutralizing HCV. It is important to note, however, that the immune globulin preparations used in these studies, though containing neutralizing antibodies, came from plasma of chronically infected patients. The role of neutralizing antibodies in viral clearance during acute infection is still unknown. Although some data suggest that the early appearance of antibodies against HVR1 after HCV infection might facilitate HCV clearance [42,43], there are no definitive data to support this hypothesis. Recent data obtained in the chimpanzee model seem to indicate that the presence of neutralizing antibodies is not necessary to obtain protective immunity [23].

There are data supporting a more relevant role of the cellular immune response for HCV clearance. One of the first studies that suggested the significance of cell-mediated immunity for HCV clearance was a report by Bjoro et al. [44] of patients with hypogammaglobulinemia who became infected with HCV. In this study it became clear that some of the patients were able to clear HCV and therefore to eradicate the virus in the absence of measurable antibodies against the virus.

Correlates of effective cellular immunity are difficult to establish and are mainly based on the analysis of CD4 + proliferative and CD8 + CTL responses during the acute phase of HCV infection in patients or chimpanzees [45–47]. In general, studies analyzing the cell-mediated immunity against HCV have described the polyclonal nature of a host immune response as well as its low vigor during chronic HCV infection [48–50]. CD4 + T-cell proliferative responses are stronger in patients resolving an infection

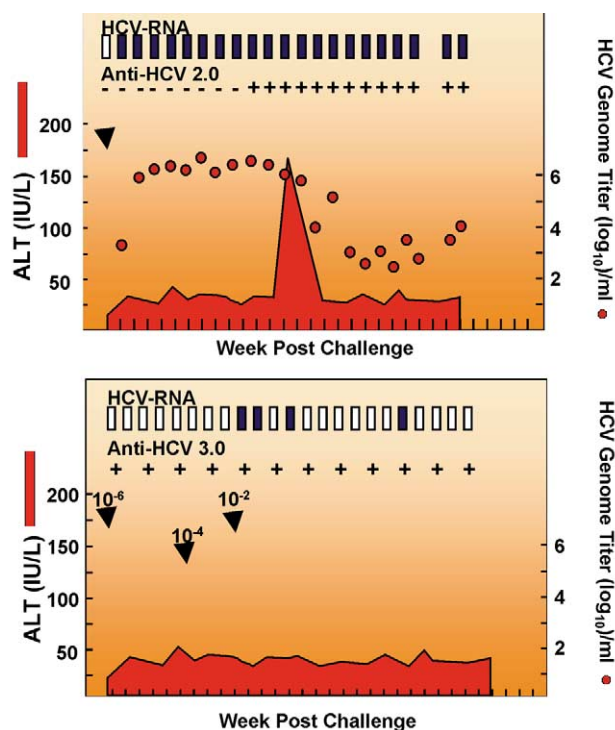
compared with those developing a chronic infection: multi-specific T-cell responses directed against both structural and non-structural antigens are more frequent and are significantly stronger in patients who clear viremia than in those who do not [47,51–54]. Furthermore, T-cells obtained from individuals with self-limiting infection display a strong profile of Th1 cytokines, such as interleukin 2 (IL-2) and interferon (IFN)-gamma, after stimulation with HCV antigen [51,55,56]. Further evidence supporting a role of Th1-type responses in contributing to viral clearance comes from the finding that strong CTL responses correlate with successful clearance of HCV infection in chimpanzees [57]. Cooper et al. [57] found that two chimpanzees with acute resolving infection had an early and strong intrahepatic CTL response to several HCV epitopes, whereas four animals that became chronically infected had much weaker CTL responses. Thimme et al. [58] have recently reported that in naive chimpanzees experimentally infected with HCV, the animals that controlled the virus at low levels or had viral clearance showed strong intrahepatic CD4 + and CD8 + responses, whereas animals without control did not.

New data obtained in the chimpanzee model suggest that protective immunity against HCV can be elicited [59]. In fact, chimpanzees that had previously cleared HCV infection were able to clear HCV rapidly following re-challenge with homologous or heterologous HCV [60,61] (Fig. 2). Strong peripheral and intrahepatic CD4 + responses appear to be associated with this protective immunity. However, in at least one case, HCV was able to persist following homologous re-challenge in an animal that had previously cleared the exact same virus, despite a strong anamnestic cellular immune response (JB, unpublished data).

### 2.4. Technical limitations: lack of reproducible cell-culture systems and small animal models

There are several technical limitations that make the development of an HCV vaccine difficult. The evaluation of the neutralization activity of antibodies to the envelope proteins could be a crucial step for the development of a protective HCV vaccine. Even though such antibodies might not play a crucial role during the natural acute infection, they might be very important for generating effective vaccine-induced immunity. However, the lack of a reproducible cell culture system and of a small animal model that supports HCV replication is one of the major obstacles for the development of such assays. HCV has been shown to replicate at low levels in human continuous T and B cell lines [62,63]. Although these cell lines have been used to develop a classic neutralization assay, infection of the cells is not consistent, which makes the interpretation of the data very difficult.

The lack of a classical neutralization test based on cell culture systems has prompted many researchers to develop surrogate neutralization assays. In developing such indirect neutralization assays it should be considered that HCV



**Fig. 2.** Previously infected and recovered chimpanzees control HCV replication after rechallenge. (A) A naive chimpanzee challenged with the lowest infectious dose of monoclonal HCV developed a chronic infection. Viral RNA appeared in serum at week one postinoculation and remained detectable by a commercially available assay through follow-up. Serum liver enzyme levels increased above normal levels at week 15. Anti-HCV became detectable at week 12 (adapted from ref. [91]). (B) A chimpanzee that had been previously infected with HCV and recovered from infection was challenged with serial dilutions of plasma from a chimpanzee infected with a monoclonal homologous HCV. The animal became HCV-RNA positive only after receiving a  $10^{-2}$  dilution; the infection was characterized by low level intermittent viremia (detectable only by a sensitive in house nested polymerase reverse transcriptase chain reaction assay) (adapted from ref. [61]). Reverse-transcriptase-nested polymerase chain reaction for HCV-RNA ■ positive, □ negative, ▼ time of challenge.

neutralization involves blocking of attachment or post-attachment events after interaction of envelope protein (E1 and E2) with the host cell. Therefore, most of the efforts are concentrated on these proteins. Rosa et al. [64] developed a quantitative test to estimate the ability of anti-HCV to block the binding of recombinant HCV E2 protein to a human T-cell lymphoma cell line (MOLT-4). Sera from chimpanzees vaccinated with recombinant E1/E2 (see below) were tested in this assay and high ‘neutralization of binding’ titers correlated with protection from infection following HCV challenge. Recent experiments showed that HCV envelope E2 protein binds CD81, a tetraspanin that is expressed on many cell types, including hepatocytes and B-lymphocytes [65]. The authors demonstrated that recombinant E2 could bind human CD81 on cells. It was also demonstrated that HCV particles could bind recombinant CD81. Therefore, CD81 has been proposed as the putative

HCV receptor. Sera from vaccinated chimpanzees protected against HCV challenge were able to inhibit the interaction of HCV and CD81. Based on these data, an assay capable of measuring neutralization of E2-CD81 binding is currently used as a surrogate neutralization assay. It is important to note, however, that neutralization by inhibition of contact between the viral attachment site and its cell receptor occurs only rarely. In fact, neutralization by blocking un-coating of enveloped viruses or by blocking of a stage of infection subsequent to primary un-coating is thought to be a more common mechanism of virus neutralization than neutralization of binding [66].

More recently, Takikawa et al. [67] developed an assay that measures the cell fusion activity of the envelope proteins of HCV. Chimeric E1 and E2 proteins, each containing its envelope ectodomain and the transmembrane and cytoplasmic domains of the vesicular stomatitis virus G protein, were expressed on the cell surface. Cells expressing the chimeric proteins were co-cultured with various cell lines transfected with a reporter plasmid. Blocking of the fusion activity, which was found to depend on the presence of both E1 and E2, could potentially be used as a measure of neutralization.

The chimpanzee model is the only animal model that can be used to test the efficacy of HCV vaccine candidates. However, the chimpanzee model is very expensive and of limited availability, and it would therefore be desirable if studies with related viruses in a smaller animal model could provide data that are of help in designing vaccine strategies for HCV. In the HIV research field, studies of the related SIV in macaques have played a significant role in defining immunity and for designing strategies for vaccine development (reviewed in [68]). It is possible that infection of tamarins with GBV-B could similarly serve as a surrogate animal model for infection of chimpanzees or humans with HCV. In a recent study, it was demonstrated that, as with HCV infections of chimpanzees, the immunity following resolved GBV-B infection in tamarins was not dependent on neutralizing antibodies (JB, unpublished data). Thus, studies of relevance for HCV vaccine development could perhaps be performed with GBV-B in tamarins.

The recent development of HCV replicons has been a major breakthrough in the field of HCV research [63,69]. Subgenomic selectable RNAs of HCV have been shown to self-replicate to high levels in the human hepatoma cell line Huh-7, which allows not only for the study of protein function, but also for testing of antiviral drugs. Although there has still not been success in the production of viral particles after the incorporation of the HCV structural proteins in the replicon system [70], this system permits expression of the envelope proteins as heterodimers that might better represent the native confirmation of these surface proteins. Also, having a cell line that replicates full-length HCV RNA opens up new avenues for further attempts to develop a true cell culture for HCV and it is anticipated that this would represent an extremely useful tool for vaccine development.

**Table 1**  
**Current approaches to HCV vaccine development**

Vaccine approach	Vector/HCV genomic region	Animal model	Humoral response	Cellular response	Optimization strategies
Recombinant E1–E2	E1/E2	Chimpanzees	Strong	Not tested	Truncated E2 (secreted form)
Virus-like particles	Recombinant baculovirus containing C–E1–E2	Mice, guinea pigs	High titers of antibodies against E2	Strong, Th1-type	
DNA vaccine	Structural and non-structural genes	Mice, tamarins, rhesus monkeys, chimpanzees	Moderate	Strong, better using non-structural regions. Predominantly Th1-type response	Modification of antigen presentation, site of DNA injection, stimulatory CpG sequences, other adjuvants such as IL-2
Recombinant viruses	Adenoviruses or vaccinia recombinants containing structural or non-structural genes	Mice	Moderate	Strong, Th1-type	Administration of recombinant viruses expressing adjuvants such as IL-2. DNA priming, vaccinia recombinant boosting
Peptides	Recombinant E1/E2 followed by HVR1 peptide; HVR1 mimitopes	Mice, chimpanzees	Strong	Not tested	
Attenuated bacteria	Salmonella containing NS3 gene	Mice	None	Strong CD8 + T cells, Th1-type	

### 3. Approaches to HCV vaccine development

The classical approaches to vaccine development, live attenuated or whole inactivated virus, are not feasible for HCV because there is no cell culture system to produce viral particles. In addition, a live attenuated approach is not realistic because of the high tendency of the virus to persist in the host. Even recombinant viruses, such as those with deletions of the 3' untranslated region or HVR1, which appear to cause attenuated acute disease in chimpanzees, readily persist in the host [31,71]. Thus, efforts to develop a hepatitis C vaccine have been primarily based on production of recombinant proteins and on DNA-based immunization (Table 1).

#### 3.1. Recombinant proteins

Envelope proteins (especially E2) have been chosen as an important target for HCV vaccine development, since they are likely to be involved in virus-host recognition and antibodies directed to these proteins appear to neutralize HCV. The first attempt to develop an HCV vaccine was by Choo and coworkers [72,73]. Vaccination of naïve chimpanzees with mammalian cell-derived E1E2 along with an oil/water adjuvant prevents the development of chronic infection, following experimental challenge, with either homologous HCV-1 (10/12 protected versus 3/10 in controls;  $P = 0.02$ ) or heterologous HCV-H (9/10 protected versus 3/10 in controls;  $P = 0.02$ ) (Table 2). In combination, 19/22 vaccinated chimps were protected from the development of chronic infection versus 6/20 in the control, unvaccinated group ( $P = 0.001$ ) (M. Houghton, personal communication). No correlation was found between the detection of anti-HVR1 and protective immunity [74]. Although the

challenge dose used in this study was low [ten chimpanzee 50% infectious doses ( $CID_{50}$ )], it suggests that antibody-mediated protection against a homologous strain of HCV is possible after immunization with recombinant subunit vaccine. Furthermore, cellular immunity elicited by the recombinant protein vaccine might have facilitated clearance of HCV in animals that became infected. However, it remains to be determined whether this vaccine provides any protection against higher challenge doses or against challenge with viruses of other subtypes or genotypes. If this is not the case, the technical difficulty involved in the production and purification of recombinant HCV envelope proteins from mammalian cells becomes a serious problem for developing polyvalent protein subunit vaccine candidates against HCV.

Recombinant envelope 1 (E1) protein might also have potential as an HCV vaccine candidate. In this regard, it is noteworthy that a candidate therapeutic vaccine for chronic hepatitis C infection, based on the E1 protein, was recently developed and tested in infected chimpanzees and healthy human volunteers. Immunization of chronically infected chimpanzees with the candidate E1 vaccine resulted in a significant improvement of the necro-inflammatory changes in the liver and a marked decrease in the expression of HCV proteins in infected hepatocytes, despite the persistence of HCV-RNA in the serum [75]. In human volunteers, three to four vaccine doses induced a demonstrable humoral anti-E1 response and a strong and specific cellular anti-E1 response of Th-1 type [76]. The study of the efficacy of this E1 protein as a prophylactic HCV vaccine candidate in the chimpanzee model is needed.

The use of virus-like particles as vaccine candidates is an attractive approach because they might mimic more closely the structure of native viruses. A potential obstacle for their use

as a vaccine candidate may be the difficulty in the generation of sufficient amounts of particles. As stated above, expression of envelope HCV proteins in mammalian cells as well as the generation of stable cell lines producing E1 and/or E2 is difficult. A recent study reported the synthesis of HCV-like particles in insect cells using a recombinant baculovirus containing cDNA encoding the HCV structural proteins (C, E1 and E2) [77,78]. The virus-like particles exhibited biophysical and antigenic features similar to the putative HCV virions. The non-infectious particles were used to immunize mice and guinea pigs and elicited a strong humoral response in both species [77–79]. Anti-envelope antibodies from immunized mice recognized E2 proteins from different HCV genotypes. In addition, HCV-like particles elicited a strong cellular immunity in mice, predominantly of the Th-1 type. Furthermore, these immune responses appear to be protective against infection with recombinant vaccinia virus expressing HCV antigens in a mouse model [80]. Additional studies in the chimpanzee model are urgently needed to establish the relevance of this attractive approach.

### 3.2. DNA vaccines

DNA-based immunization induces humoral and cellular immune responses by synthesis of viral antigen *in vivo* [81]. Since cellular immunity appears to be relevant for HCV

clearance, this might represent a theoretical advantage over protein-based vaccines, which in general are less efficient in generating cellular immune responses. Another potential advantage of this type of immunization with regard to humoral immune responses is that post-translational modifications of the antigen occur within the cell and most likely mimic native protein folding. In addition, manipulation of DNA provides the opportunity to modify its presentation to the immune system by changing its cellular location or increasing its immunogenicity. Also, this method of immunization makes it feasible to develop polyvalent vaccine candidates since the manipulation of multiple different DNA molecules would be simpler than the purification of multiple different proteins. Importantly, DNA-based immunization has been able to confer protection against challenge with other viruses [81].

Previous studies have shown that DNA immunization can generate antibodies against the structural proteins of HCV [82–86]. Recently, the major focus of these studies has been the E2 protein because it is believed that E2 contains important neutralization epitopes. Vaccine candidates should mimic as closely as possible the native conformation of E1/E2 heterodimers, thought to represent functional subunits of HCV virions. In general, plasmids encoding full-length forms of E1 or E2 are poorly immunogenic [82–84], as are constructs designed to produce interactive E1

**Table 2**  
Vaccine candidates tested for protective immunity in the chimpanzee model

Recombinant subunit vaccine (E1–E2 heterodimers) ([72,73]; Houghton, personal communication)					
Homologous polyclonal challenge (HCV-1; genotype 1a)	Dose	Complete protection	Self-limited acute infection	Acute hepatitis (ALT elevation)	Chronic infection
Vaccines ( <i>n</i> = 12)	10–100 CID	5	5	7 (enzyme elevation lower than controls)	2
Controls ( <i>n</i> = 10)	1–100 CID	0	3	10	7
Heterologous polyclonal challenge (H77; genotype 1a)	Dose	Complete protection	Self-limited acute infection	Acute hepatitis (ALT elevation)	Chronic infection
Vaccines ( <i>n</i> = 10)	10–100 CID	0	9	10 (enzyme elevation lower than controls)	1
Controls ( <i>n</i> = 10)	10–64 CID	0	3	10	7
DNA vaccine (E2) ([91]; JB, unpublished data)					
Homologous monoclonal challenge (H77C; genotype 1a)	Dose	Complete protection	Self-limited acute infection	Hepatitis	Chronic infection
Vaccines ( <i>n</i> = 2)	100 CID	0	2	2	0
Controls ( <i>n</i> = 2)	3–64 CID	0	0	2	2
Recombinant subunit vaccine (E1/E2) and peptide vaccine (HVR1) [113]					
Polyclonal challenge (HCV-#6; genotype 1b)	Dose	Complete protection	Self-limited acute infection	Hepatitis	Chronic infection
Vaccine ( <i>n</i> = 1)	10 CID	1 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0
Control ( <i>n</i> = 1)	10 CID	0	1	1	0

<sup>a</sup> The animal developed an acute self-limited infection following vaccination with E1/E2 and subsequent heterologous challenge. Once the animal was boosted with HVR1 peptides and the anti-HVR1 titer increased, complete protection was achieved against homologous polyclonal challenge.

and E2 [87]. One of the strategies used to increase the immunogenicity of E2 has been to modify its presentation to the immune system, by favoring secretion or cell-surface expression. Secreted forms of E2 were obtained by removal of its hydrophobic carboxy-terminal domain and cell surface-expressed E2 was obtained by exchange of this domain with a membrane anchor. In vitro experiments based on the recognition of modified E2 by conformation-sensitive antibodies and binding to the putative HCV receptor CD81 suggest that E2 can be modified without significant alteration of its folding [88]. Both a secreted and a cell-surface expressed E2 elicited relatively strong immune responses in mice, tamarins and rhesus macaques [82,87] (Fig. 3A). In general, E2-based DNA immunization generates a Th1-type cellular immune response in mice, with induction of IgG<sub>2</sub> antibody isotype and production of interferon-gamma by splenocytes [86,89]. Generation of specific CTL activity against envelope proteins after DNA immunization is however weak, but it can be stimulated by booster immunization with recombinant proteins [90].

A recent study explored the immune responses elicited in two chimpanzees by DNA immunization with a plasmid encoding a cell-surface HCV-envelope E2 glycoprotein [91]. This DNA vaccine candidate was previously shown to induce immune responses in mice and rhesus macaques [82]. One chimpanzee developed relatively high titers of anti-E2 and anti-HVR1 whereas the other vaccinated animal developed low levels of anti-E2. CTL responses to E2 were not detected before challenge but an E2-specific CD4 + response was detected in one of the animals. Three weeks after the last immunization, both chimpanzees were challenged with 100 CID<sub>50</sub> of homologous monoclonal HCV (Table 2). As controls, two naive chimpanzees were inoculated with three to 64 CID<sub>50</sub> of the challenge virus. This monoclonal virus, obtained from a chimpanzee transfected with an infectious cDNA clone of HCV, permitted challenge without the confounding factor of virus diversity (quasispecies). Even then, the vaccine did not generate sterilizing immunity as both vaccinated animals were infected and developed mild hepatitis. However, both vaccinated chimpanzees cleared the virus relatively early (less than 12 weeks) whereas the control animals became chronically infected. The results of this study suggest that DNA-based immunization with E2 modified the course of the infection and might have prevented progression to chronicity.

Although the E1 protein appears to be less immunogenic than other viral components, a recent study [92] demonstrated that mutations of the *N*-glycosylation sites could enhance the humoral immune response elicited in mice by a plasmid DNA vaccine encoding the E1 protein. More importantly, antibodies generated after immunization of mice with plasmids encoding such modified E1 proteins were able to recognize HCV-like particles. These results suggest that the de-glycosylation of the E1 protein made some epitopes more accessible to the immune system.

HCV nucleocapsid has also been used for DNA-based

immunization experiments because it elicits strong humoral and cellular immune responses after natural infection [33,83,90,93]. Mice immunized with plasmid DNA encoding the core protein developed a Th1-like immune response as shown by the cytokine profile of splenocytes from immunized mice; in contrast, mice immunized with recombinant core protein developed a predominant Th2-like response [94]. The inclusion of core sequences in a candidate HCV vaccine raises some concerns, however, since the HCV core protein has been associated with cellular proliferation, differentiation and apoptosis [95].

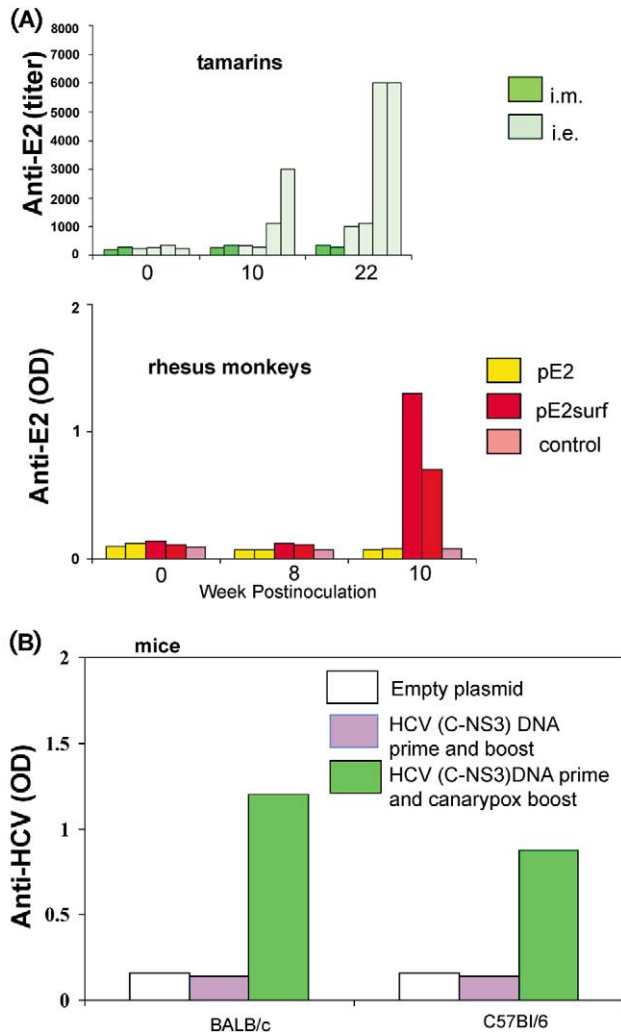
Genes encoding HCV non-structural proteins are also attractive candidates for DNA-based immunization since these proteins are targets of the cellular immune response. Several CTL epitopes have been described within NS2, NS3, NS4B, NS5A, and NS5B as well as T-helper epitopes within NS3. As stated above, vigorous CD4 + T-cell proliferative responses directed against NS3 are associated with clearance of HCV infection, both in humans and chimpanzees. Preliminary results suggest that DNA-based immunization with plasmids encoding non-structural proteins can generate strong cellular immune responses in mice [96,97].

Increased immunogenicity of DNA vaccines can be obtained by changing the route of immunization, by addition of adjuvants or by combination of different immunization schedules [90]. Coinjection of plasmids encoding HCV core with DNA encoding granulocyte-macrophage colony stimulating factor, IL-2 or IL-4 increased humoral and cellular immune responses as compared with HCV core alone [98]. The immune responses can also be improved by increasing the content of CpG motifs [99,100]. These sequences, in addition to activating B cell responses, directly activate monocytes, macrophages and dendritic cells to secrete IL-12, tumor necrosis factor-alpha and IFN alpha/beta. These cytokines stimulate NK cells to secrete IFN gamma and therefore promote a Th1-type immune response. The ability to induce strong Th1 immune responses is thought to be important for resolution of HCV infection. Other approaches being explored are the fusion of antigens with ubiquitin genes to optimize peptide transport to the MHC class I pathway, the use of tissue-specific promoters or the targeting of DNA to antigen-presenting cells [90].

DNA-based immunization has, however, some disadvantages compared to other vaccine approaches. First, DNA vaccines elicit a weaker humoral immune response in comparison to protein-based vaccines. Second, DNA-based immunization results obtained in one animal species can not be extrapolated to other species and this is especially relevant for HCV. Furthermore, the effect of immune adjuvants and routes of immunization, especially in humans, has not been well established.

### 3.3. Other vaccine approaches

The use of adjuvants is a well-known strategy to improve local and systemic immune responses. The immunostimu-



**Fig. 3.** Strategies to enhance the immune responses after DNA immunization. (A) Intraepidermal immunization of tamarins with a plasmid encoding a secreted form of the HCV E2 glycoprotein elicited a significantly stronger humoral immune response than intramuscular immunization with the same plasmid. In rhesus monkeys, a plasmid encoding a surface-expressed HCV E2 glycoprotein generated a significantly stronger humoral immune response than a plasmid encoding a full-length E2 (adapted from refs. [82,87]). I.m., intramuscular; i.e. intraepidermal; pE2, plasmid encoding a full-length E2; pE2surf, plasmid encoding a surface-expressed E2; arrows indicate time of immunization. (B) Mice immunized with a polycistronic construct encoding HCV structural and non-structural proteins followed by a boost with a non-replicating canarypox containing the same HCV genes generated potent immune responses in two different mouse strains (adapted from ref. [109]).

lating complex or ISCOM is a particulate adjuvant/antigen system that incorporates lipids into the immunization complex [101] and that has already shown some benefit when used in rhesus macaques immunized with HCV core protein [102].

Recombinant viruses are an efficient way to deliver heterologous DNA that can mediate high levels of protein expression in host cells. Some studies have already explored the immune responses generated by defective recombinant

adenoviruses containing genes from the structural proteins of HCV [93,103]. Both humoral and cellular immune responses were obtained in immunized mice; cellular immunity might be optimized by co-administration of adenovirus expressing IL-2 [93]. Similarly, strong CTL and CD4 + proliferative responses were obtained in mice immunized with recombinant vaccinia viruses expressing the structural proteins of HCV. Interestingly, when core sequences were included in recombinant vaccinia viruses, a significant suppression of specific vaccinia CTL responses occurred [104], suggesting that inclusion of core sequences within a vaccine candidate might decrease immune responses directed against other antigens.

Besides the use of adenoviruses and vaccinia recombinants, new strategies include the use of new poxvirus vectors (canarypox, fowlpox) [105], attenuated vaccinia virus strains (Ankara) or alphavirus (Venezuelan equine encephalitis) [106,107]. Similarly, recombinant Semliki Forest virus particles expressing HCV NS3 were capable of inducing a strong NS3-specific cellular immune response in transgenic HLA-A2.1 mice; the cellular immune response targeted a dominant HLA-A2 epitope previously described in infected patients [108]. The use of recombinant poxvirus bearing HCV genes to boost the immune response after priming with DNA or protein seems to be a promising approach [109] (Fig. 3B). Recently, vesicular stomatitis virus recombinants expressing high levels of HCV E1 and E2 proteins were constructed; these viruses could also be used to generate immune responses against HCV envelope proteins [110]. Live attenuated Salmonella bacteria have also been used as a vehicle for DNA delivery and have successfully generated immune responses against HCV in mice [111,112].

As the HVR1 of the HCV E2 protein, a short region consisting of only 27 amino acids, contains a neutralization domain and anti-HVR1 antibodies can neutralize HCV [22], a peptide vaccine approach is also being explored. In a study by Esumi et al. [113] (Table 2), a chimpanzee was vaccinated with recombinant E1 and E2 proteins as well as with peptides encoding epitopes within E1 and E2 (including the HVR1) deduced from a genotype 1b isolate. Although anti-E1 and anti-E2 responses were good following immunization, the animal developed a self-limiting infection after heterologous HCV genotype 1b challenge. The chimpanzee was thereafter immunized with an HVR1 peptide that represented the dominant sequence in the challenge pool; a significant increase in anti-HVR1 titer was observed and the animal did not become infected following a new low dose homologous challenge. Sera taken immediately before challenge could neutralize the homologous virus in vitro. This study suggests that generation of antibodies against linear epitopes (HVR1) using a peptide vaccine might be a useful approach for vaccine development, although the extreme genetic heterogeneity of this region represents a major challenge. One approach to circumvent this problem is to select highly cross-reactive 'synthetic' HVR1 peptides

(mimotopes); these unique variants can induce antibodies that interact with a large number of naturally occurring HVR1 sequences. After immunization of mice with different candidate ‘mimotopes’ it was found that the ones with the broadest cross-reactivity induced antibodies which recognized the HVR1 peptides deduced from many different HCV variants [114]. Monoclonal antibodies obtained from immunized mice were capable to bind HVR1 contained in a soluble form of E2 and more importantly, one of these monoclonal antibodies was capable to capture viral particles and recombinant HCV-like particles assembled in insect cells [115]. It remains to be determined whether such mimotopes also induces cross-reactive antibodies in the chimpanzee model.

#### 4. Summary

It is apparent that the development of an HCV vaccine poses a great challenge to the scientific community. Some difficulties are inherent to the virus, such as its high genetic heterogeneity and the ability to establish persistent infections, perhaps by escaping the host immune responses. Other limitations are technical, such as the lack of a cell culture system or a small animal model for HCV. Several studies have demonstrated that neutralizing antibodies to HCV exist, but that they appear to be isolate- or strain-specific and thus, a vaccine capable of generating sterilizing immunity is a challenge. Studies performed in humans and chimpanzees suggest that resolution of HCV infection is associated with a strong cellular immune response. Since hepatitis C virus causes the most serious liver damage after an extremely protracted course, a vaccine that generates immune responses capable of converting an evolving persistent infection into a self-limiting infection represents a reasonable goal and would have major impact on the disease caused by HCV infection.

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