Development of a hepatitis C virus vaccine

Genevieve Inchauspé, PhD\textsuperscript{a,\*}, Stephen Feinstone, MD\textsuperscript{b}

\textsuperscript{a}Unité Mixte CNRS-BioMérieux, UMR 2142, Ecole Normale Supérieure, 46 Allée d’Italie, Lyon Cédex 17-6934, France

\textsuperscript{b}Laboratory of Hepatitis Viruses, Center for Biologics Evaluation and Research, US Food and Drug Administration, Bldg. 29A/Room 1D14, Bethesda, MD 20892, USA

Any program aimed at the development of a vaccine should consider several important issues because they may greatly influence the choice of immunogen used in the vaccine, the delivery system selected for its application, the population to be vaccinated, and the type of vaccine to be developed (ie, preventive or therapeutic). These issues concern the epidemiology of the infectious disease targeted, the actual routes of transmission, the antigenic diversity of the infectious agent, the existing therapies, and their rate of success. In the case of hepatitis C virus (HCV), a viral agent whose clinical existence was recognized in the 1970s but which was only identified by the use of molecular cloning technology in the late 1980s [1], some of these issues are particularly relevant. For example, existing therapies are in constant evolution, a fact bound to influence the selection of populations that may benefit from a therapeutic vaccination.

Prevalence and transmission

HCV infections are distributed worldwide. About 170 million people are estimated to be infected, representing about half of the number believed infected with hepatitis B virus (HBV) but approximately 7 times more than the number of HIV carriers (Table 1). The overall seroprevalence of anti-HCV in different populations around the world is estimated to be about 3%. Though there were an estimated 150,000 new cases of HCV infection each year in the United States before the implementation of blood screening tests and altered behavior caused by AIDS awareness [2], HCV infection has now dropped to 33,000 new cases yearly, representing about 12% of all case of viral hepatitis. Whereas reduction in
the number of new infections is a general phenomenon in countries that have installed the systematic screening of blood donations, transmission of HCV in these countries is far from negligible and still represents a serious medical and economic challenge. One controversial route of transmission is the sexual route. A number of studies have failed to demonstrate evidence of HCV infection in spouses of carriers, though others have confirmed that transmission between spouses can occur [3]. The risk of mother-to-infant transmission is highly variable but lower than 10% with an average 6% [4]. There seems to be no risk when HCV RNA is absent, but the risk tends to increase with HCV viral load and with HIV coinfection (see the article by Dr. Thomas elsewhere in this issue).

Globally, high-risk populations include hospital workers, dialysis patients, organ recipients, relatives of infected carriers, and intravenous (IV) drug users who represent the group with the highest risk of transmission [2].

**Genotypic diversity**

There is no serotypic classification of HCVs for the simple reason that there is no available test to allow such classification. The lack of an efficient, reproducible in vitro replication assay has rendered the performance of cross-neutralizing tests to define serotypes virtually impossible. Cross-challenge experiments performed in chimpanzees, the only existing susceptible animal model, have yielded observations difficult to interpret. Indeed, chimpanzees that have been exposed to a viral challenge and that recovered from infection are generally susceptible to a new challenge, whether homologous or heterologous [5]. The lack of protective immunity, even to a homologous strain, shows that serologic differences between HCV strains remain undefined.

A major feature of HCV and a leading concern for vaccine development is the heterogeneity of its genome (see the article by Dr. Pawlotsky elsewhere in this issue). Mutations, resulting from the typical error-prone RNA-dependent RNA polymerase and lacking proofreading activity common to RNA viruses as well as from the host immune pressure, are responsible for the fact that HCV circulates in the host as a complex viral population referred to as a quasispecies [6]. The existence of quasispecies could be one mechanism used by HCV to evade the host-immune surveillance. The nature and complexity of such quasispecies may

---

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>HCV</th>
<th>HBV</th>
<th>HIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global prevalence</td>
<td>170 M</td>
<td>1.2 Bil</td>
<td>36.1 M</td>
</tr>
<tr>
<td>Chronic infection</td>
<td>129 M</td>
<td>350 M</td>
<td>36.1 M</td>
</tr>
<tr>
<td>Deaths/year</td>
<td>476,000</td>
<td>1.2 M</td>
<td>2.8 M</td>
</tr>
<tr>
<td>Annual death rate</td>
<td>0.4%</td>
<td>0.49%</td>
<td>7.8%</td>
</tr>
<tr>
<td>Lethality</td>
<td>7–10%</td>
<td>—</td>
<td>(100%)</td>
</tr>
</tbody>
</table>


*Abbreviations:* HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immune deficiency virus.
also influence the response rate to therapies. Some studies have shown that patients with a more homogeneous viral quasispecies appear to be better responders to interferon (IFN) therapies than those who display a relatively more complex one [7]. Extensive phylogenetic analyses has permitted classification of HCV isolates into 6 major genotypes (1–6) containing at least 70 different subtypes (a, b, c) [8]. HCV genotypes 1, 2, and 3 are the most widely encountered around the world. Globally, genotype 1 is most prominent in North America and Europe (subtype 1a) as well as in Asia (subtype 1b). The prevalence of genotypes 2 and 3 is also significant in North America, Europe, and Asia, whereas genotype 4 is the predominant form in North and Central Africa. Genotype 5 has so far been mostly identified in South Africa, and, to date, genotype 6 isolates have been found primarily in Vietnam and Hong Kong.

Actual therapies and response rates

The application of a therapeutic vaccine, at first, for obvious ethical reasons, targets patients who have failed a therapeutic intervention. It is thus of upmost importance to try and: (1) establish markers predicting a response in order to administer the candidate vaccine as early as possible after initiation of therapy; and (2) identify the relationship between response rate to treatment and the infecting genotype in order to select the most appropriate genotypic sequences to put in the vaccine. Latest available data indicate that the efficacy of the consensus therapy used today, a combination of pegylated interferon-alpha (IFN-α) and ribavirin, is greatly dependent on the infecting genotype. Long-term response rates can approach 80% for genotypes 2 and 3 but as low as 40% to 45% for genotype 1a/b or 4 [9]. Thus, these poorly responsive viral genotypes may represent the target of choice of the first generations of therapeutic vaccines. One might assume the greatest likelihood for a vaccine to demonstrate a positive impact would be early after treatment failure. A recent report by Jessner et al [10] showed that the measure of the viral load decline at 24 hours after the onset of therapy predicted the eventual response of the treated patient. If confirmed, these measurements may help to optimize therapeutic vaccine applications.

Immune correlates of resolution and persistence

Following infection by HCV, a minority of patients develop symptoms during the acute phase and manage to clear the virus. Such viral clearance has been attributed to the development of an early, vigorous, and polyclonal T cell response that persists in the host (see article by Dr. Chang) [11–13]. Specific CD4+ and CD8+ T cell responses have been documented that target multiple epitopes spanning basically all of the HCV antigens. Enhanced T cell-mediated responses following therapeutic resolution have been observed, although these responses apparently
target different cytotoxic T lymphocytes (CTL) epitopes than those observed in nontreated patients who clear the virus [14]. No specific antigens or epitopes have so far been linked to these natural or therapeutic resolutions, with the exception of a specific T-helper epitope and a dominant CTL epitope both located in the nonstructural protein 3 (NS3) [14,15]. Intensive efforts are currently being pursued to identify other such epitopes that could be important components of a vaccine.

The presence of detectable virus-specific CTL in the blood or the liver of chronically infected patients has been documented [11,16,17], but their frequency appears to be much lower than that observed in acutely resolved infections [18]. A vigorous initial CTL response after a recent infection, as measured by tetramer analysis, takes place although it has been described as short-lived [19]. The mechanisms involved in the brevity of such response and its inefficient nature are as yet unclear. Emergence of viral variants has been reported in chimpanzees and humans. A recent study demonstrates that an early escape from immunodominant CTL epitopes is likely to be a critical determinant of HCV persistence [20]. Intriguing also are reports describing the presence, during chronic infection, of HCV sequences in dendritic cells together with an alteration of the allostimulatory function of these cells [21,22]. Infection of dendritic cells (DC) by HCV and the consequent dysfunction of these cells, which constitute key players in the mounting of a specific immune response, may be a major issue to consider in the development of a therapeutic vaccine.

Though mounting evidence is being accumulated that points to a major role played by the cellular immune response in the control of an HCV infection, that of the existence and efficacy of neutralizing antibodies is much less clear. It has clearly been shown that it is possible to neutralize HCV in vitro [23–25], and chimpanzee vaccine/challenge studies also suggest that serum antibody may be important in protection from or attenuation of infections [1] (see Table 2). Two recent major studies, one performed in chimpanzees and one in humans, appear to rule out a critical role (or any role at all) of such antibodies in the resolution of infection [26,27] because control of infection could be linked only to the mounted HCV-specific cellular immune response.

**HCV vaccines: to-date consensus**

In developed countries, it is generally accepted that the development of a preventive vaccine should benefit at least high-risk populations. In developing

<table>
<thead>
<tr>
<th>Vaccine candidates</th>
<th>Mice</th>
<th>Macaques</th>
<th>Chimpanzees</th>
<th>Humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptides</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Recombinant antigens</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>DNA</td>
<td>+++</td>
<td>–</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>Vectored</td>
<td>++</td>
<td>–</td>
<td>(+)</td>
<td>–</td>
</tr>
<tr>
<td>Prime/boosts</td>
<td>++</td>
<td>–</td>
<td>(+)</td>
<td>–</td>
</tr>
</tbody>
</table>
countries where blood donations may not be systematically screened for HCV infection and where very expensive therapies are also impractical, it is clear that the general population (in its entirety) should be a candidate for vaccination. The use of a therapeutic vaccine would be of obvious worldwide interest. Used alone or in combined regimens with existing therapies, the goal of such a vaccine should be to achieve a greater rate of resolution, to lower the cost of therapies and, hopefully, to be better tolerated than the existing drugs. Most of the focus to date, as will be illustrated in the following sections, is on the development of vaccines capable of inducing strong, cross-reactive, and long-lasting cellular-mediated responses. Though reaching such a goal would be a clear achievement, this may not be sufficient to obtain either an efficient preventive vaccine or a therapeutic one. In total absence of neutralizing antibodies, prevention of de novo infection by progeny viruses may not be achieved. In addition, unless restoration of some of the basic functions of cells from the immune systems that seem to be directly infected by HCV (such as DCs) is achieved, a therapeutic vaccine may not succeed in helping the immune system of the infected host to eliminate or even control infection. Finally, a major issue concerning therapeutic vaccines is currently under debate. It is indeed difficult to predict whether specific CTL responses in chronic HCV patients may exacerbate CTL-mediated liver injury instead of diminishing or controlling it.

**Experimental approaches**

**Preclinical studies**

**Single vaccine regimens**

**Peptide-based vaccines.** Induction of a multi-specific cellular immune response directed simultaneously against multiple HCV epitopes appears to be important for the development of an efficacious HCV vaccine. Important advances in epitope design and in the development of transgenic mouse models expressing different human leukocyte antigen (HLA) class I or class II molecules have rendered more attractive and relevant vaccine approaches based on the use of synthetic peptides. Combinations of different peptides, representing single or multiple epitopes encompassing different HCV antigens and covering different HLA molecules, should provide a rather broad population coverage. Surprisingly, this field has attracted only a limited amount of interest. Most of the work performed so far consists in the characterization of immunogenic peptides rather than the testing of peptide-optimized vaccine formulations. Major histocompatibility complex (MHC)-peptide binding assays have led to the identification of many CTL epitopes in the HCV polyprotein [4,28–30]. Attempts at enhancing immunogenicity of an epitope by modifying its primary sequence has been reported in the case of a well-conserved HLA-A2.1-restricted CTL epitope in the HCV core antigen (aa 132–140). This epitope was studied by making a series of
single amino acid-substituted nonapeptides at each amino acid position that represented the modified peptide immunogen. Peptides substituted at position 1 had a higher binding affinity but paradoxically had poorer immunogenicity. One peptide termed 8A in which the leucine at position 8 was exchanged for an alanine, exhibited both increased binding affinity and improved immunogenicity in the HLA-A2.1 mice for the wild type peptide [31].

It has been shown that induction of specific CTL following peptide-based immunization can be optimized if the peptide sequence contains not only a CD8 but also a CD4 epitope. In their study, Lopez-Diaz de Cerio [32] identified a sequence encompassing residues aa 121–135 from the E1 protein that is capable, on injection in BALB/c mice, to induce both CD4+ Th1 cells as well as CD8+ CTL. When the Val 122 residue (which likely represents an anchor residue for binding to MHC class II molecules) of the wild-type-derived sequence was replaced by Ala, complete abrogation of the CTL induction as well as production of IL2 and IFN-γ was observed. This study revealed a CD4-dependance of the aa 121–135 contained CD8+ CTL epitope. It is interesting to note, that the CTL response induced by the 121–135 E1-peptide could be obtained in total absence of adjuvant.

A novel approach for the development of epitope-based vaccines consists of using sera from HCV infected patients to screen phage-displayed peptide libraries and select peptides that specifically react with sera from infected patients [33]. Sera from HCV-infected individuals were used to screen a vast repertoire of hypervariable region 1 (HVR1) peptides expressed in a bacteriophage library to select peptides that are antigenic and immunogenic mimics of a large number of naturally occurring HVR1 variants. Mixtures of peptides with the highest cross reactivity were injected into mice and shown to induce high cross-reacting responses to most (95%) of the natural HVR1 peptides present in a panel of 40 peptides [34].

Protein-based vaccine. Evaluation of protein-based vaccine candidates has been reported in mice, macaques, and chimpanzees. The first antigens selected were HCV-encoded glycoproteins because of their presumed capacity to induce neutralizing antibodies. In a first study, Heile et al [35] compared the immunogenicity of different forms of E2 expressed in mammalian cells and purified from the core-glycosylated intracellular fraction or after secretion from the supernatants of cultures. Only the intracellular-derived proteins were able to bind to MOLT-4 cells efficiently and elicit so-called neutralization of binding (NOB) antibodies able to block the binding of E2 to the CD81, a cell-surface glycoprotein that had been shown to bind E2 and perhaps HCV, though it is not likely to be the cellular receptor for HCV (see the article by Polyak elsewhere in this issue) [36]. Compared with E2-DNA vaccines, the monomeric protein purified from the intracellular fraction, induced higher anti-E2 titers. This study underlines the fact that quantitatively and qualitatively superior anti-E2 responses are obtained with a nonsecreted form of E2. In a different approach, virus-like genomeless particles (HCV-like particles (LP) or HCV-LPs) have been produced in insect cells using a recombinant baculovirus system expressing the HCV
structural proteins (Core, E1, and E2). Gradient density analysis showed particles sediment similar to HCV virions. Upon vaccination with HCV-LPs formulated in Freund’s adjuvant, mice developed high titer anti-HCV antibodies directed against various regions of the structural proteins [37]. Interestingly, the anti-envelope antibody titers obtained in the vaccinated mice were found to be in the range of that observed in HCV-infected humans. In a second study, the authors compared the immunogenicity of HCV-LPs containing or not containing the P7 protein (located downstream of E2). Particles generated without P7 induced a stronger cellular-mediated immune response than HCV-LPs prepared in the presence of P7 [18]. The antibody isotypes as well as the detection of IFN-γ produced by the splenocytes of vaccinated mice indicated that vaccine-induced response was of a Th1 type. One major drawback to this approach so far is the fact that purity of the HCV-LP preparations remains below 10%, which restricts testing in larger animals and in humans.

When the natural immunogenicity of a protein is weak, it may be possible to enhance it by expressing the protein in fusion with a heterologous protein that has original biologic properties such as the capacity to assemble and form particles or be secreted (eg, the HBV surface antigen). One recent study has exploited the ability of the anthrax toxin lethal factor (LF) to enter the cytosol via extracellular routes to attempt delivery of HCV epitopes into cells [38]. A LF-fusion protein containing a core-derived CTL epitope was engineered and used to vaccinate mice. Whereas the immunogenicity of the purified recombinant antigen proved rather low, when dendritic cells pulsed in vitro with the preparation were administered to mice, vigorous specific CTL could be detected. These preliminary results are certainly interesting, but it clearly remains to be shown that LF-fusion proteins containing larger HCV immunogenic domains and/or multiple epitopes can be generated in order to induce the broad immune response that will be required for the development of an effective vaccine.

Because the HCV core protein is the most conserved among HCV genotypes, it has been selected by Polakos et al to evaluate the adjuvant effect of nonclassical ISCOMs (CSL Ltd., Parkville, Victoria, Australia) [39]. For that purpose, ISCOMATRIX of about 1 nm in size were used to adsorb the purified antigen on the surface. This is in contrast with a classical ISCOM formulation that has a typical size of 40 nm and in which antigens are commonly entrapped. This core-ISCOM prototype vaccine was able to prime, in macaques, strong CD4+ and CD8+ T cell responses efficiently. This vaccine elicited a Th0-type response as well as antibodies. Intracellular staining of cytokines revealed that up to 0.71% and 2.21%, respectively, of the circulating CD4+ and CD8+ were specific for naturally processed antigens. To date, this original and promising approach is being extended to HCV nonstructural antigens.

A series of vaccination experiments has been performed by the Chiron group (Chiron Corp., Emeryville, CA) in chimpanzees. Both the E1 and E2 glycoproteins were expressed in HeLa cells by a recombinant vaccinia virus. The glycoproteins were purified and mixed with an oil/water microemulsified adjuvant and inoculated IM into seven chimpanzees in varying doses and
schedules. Two to three weeks after the final boost, the chimpanzees were challenged with 10 chimpanzee infectious doses (CID50) of HCV-1 (genotype 1a), which was the same virus from which the vaccine had been made. Five animals that had the highest anti-E1/E2 responses were completely protected from infection by the challenge virus. The two low responders became infected, but both resolved their infections. This was in contrast with four unvaccinated but challenged chimpanzees that all became chronically infected [40] though the reported rate of chronicity in HCV-infected chimpanzees is about 30%. In later studies, a constitutive high E1/E2 expression Chinese hamster ovary (CHO) cell line was used as the source of the antigen for further chimpanzee studies. Of four vaccinated and challenged chimpanzees, three developed mild, short-lasting infections and one developed a persistent infection. The five protected animals from the original experiment were reboosted with the CHO cell-derived E1/E2 and rechallenged with 10 CID50 of a homotypic though heterologous strain of HCV (HCV-H, genotype 1a). In this case, all five animals were infected, though none developed persistent infections (Houghton et al, personal communication). In summary, a total of 12 chimpanzees were vaccinated, 5 were protected from low-dose challenge with a virus identical to the vaccine, and 6 developed relatively mild, short-lasting infections, and 1 developed a chronic infection. Five previously protected chimps were infected by challenge with a different isolate of a homotypic virus. These experiments seem to show that antibody to the envelope glycoproteins induced by a subunit vaccine can have some effect on the outcome of a subsequent infection to reduce the severity of the acute infection and reduce the likelihood of chronicity, though cellular immune responses were not studied in these animals. In addition, these studies did not address the issue of the possibility of multiple antigenic types of HCV.

Chimpanzees have also been used to study the potential for an immunotherapeutic approach to the treatment of chronic HCV infections. The vaccine consisted of a recombinant E1 glycoprotein of an HCV genotype 1b isolate expressed in mammalian cells. The E1 was purified to homodimers and left to associate into monodispersed spherical particles averaging 9 nm in diameter. Two long-term chronically infected chimpanzees, one with genotype 1a and the other with 1b, were inoculated with a total of nine 50 ug doses of the E1 vaccine. During the period in which the vaccines were administered, the alanine aminotransferase (ALT) levels decreased, liver histology became normal, and HCV antigen virtually disappeared from the liver. There was no change, however, in the HCV RNA levels in the serum as measured by reverse-transcriptase (RT-PCR). After the vaccine was stopped, inflammatory changes and HCV antigens reappeared in the liver, and the serum ALT levels rose to pretreatment levels. Though the animals were not cured, there was a measurable improvement in the liver disease associated with this chronic infection (unpublished data). This vaccine has completed a phase I clinical evaluation (see below).

DNA-based vaccines. HCV DNA-vaccines have recently been reviewed [41]. This section will illustrate advances in this field by describing the most significant studies performed as well as the most recent ones. Because of their ease of
construction, a wide variety of DNA vaccines have been generated that target virtually all of the viral antigens. Four major types of core-DNA vaccines have been tested. They express: (1) core under classical or specialized promoters; (2) full-length core or subgenomic domains in fusion with various regions of the secreted hepatitis B virus protein; (3) core in fusion with proteins allowing for its routing to specific subcellular compartments; and (4) core in presence of various coexpressed cytokines. Basically, all of these approaches have been able to elicit specific CD4+- and CD8+-mediated immune responses, whereas that of antibodies has been much harder to observe in part probably because of the very basic, nonsecreted nature of the protein. Only Geissler et al [42]—who coinjected GM-CSF (granulocyte macrophage-colony stimulating factor), IL2- and IL4-expressing plasmids with core DNA—showed that this approach could result in a two- to threefold increase in anticore antibody seroconversion rates. The most easily detectable immune response is that of specific core-CTLs. Using a wild-type CMV-driven core expressing plasmid, these appear to target preferentially one dominant epitope [43]. In spite of this apparent narrow scope of recognition, immune protection has been demonstrated by Tokushige et al [44] against a tumor challenge in Balb/c mice immunized with a wild type construct. Attempts to optimize the core specific T-cell responses using DNA vaccines expressing either the core sequence in fusion with the ubiquitin gene or the core sequence in fusion with the signal sequence and transmembrane domain of the murin lysosome-associated membrane protein (mLAMP-1) have so far failed [45]. This is in contrast with results obtained with similar strategies in the papillomavirus HPV-16 or the HIV models. The reasons for this failure are unclear but were also observed in the case of the NS3 protein [46].

The first generations of E2-encoding DNA vaccines have successfully demonstrated the induction of anti-E2 antibodies and T-cell responses. Plasmids typically expressed wild-type E2 sequences or discrete domains fused to the hepatitis B surface antigen, which allowed the mapping of immunogenic domains on E2 [47,48]. Subsequent studies tried to potentiate vaccine-induced anti-E2 responses by directing expression to the cell surface [49] or via the coinjection of a plasmid expressing the GM-CSF [50]. A C-terminal truncated E2 fused with the transmembrane domain of the platelet-derived growth factor receptor (PDGFR), permitting its cell surface expression was used to immunized mice and macaques [49]. Animals developed an earlier and stronger anti-E2 antibody response than animals vaccinated with a wild-type E2 expressing plasmid, but overall titers remained rather low. Lee et al [50] used a bicistronic plasmid expressing both a C-terminally truncated E2 (or E1) and GM-CSF and also tested a plasmid expressing a GM-CSF envelope fusion protein. The plasmid coexpressing E2 with GM-CSF induced the strongest responses compared with when the GM-CSF and the envelope proteins were codelivered in separate plasmids. The lowest responses were observed in animals immunized with the plasmid expressing GM-CSF envelope fusion proteins.

Though results with DNA-based vaccines were encouraging, they were only partially satisfactory as mounting evidence in the literature suggested that HCV glycoproteins E1 and E2 interact in a complex and critical manner necessary
for their proper folding. Fournillier et al have recently addressed this issue [51]. Mono- and di-cistronic plasmids expressing various forms of E1 or E2 that have retained the ability to form the noncovalent complexes or, in contrast, that have lost it, were engineered and evaluated in mice. Overall, data indicated that only when a severely truncated form of E2 was expressed could specific antibodies be observed. All constructs designed to produce interactive E1 and E2 failed to induce detectable antibodies (this was independent of the secretion levels of the antigens expressed). Finally, in this study, an absence of detectable (or very limited) anti-E1 antibodies were observed independent of the plasmid used. Thus, two major conclusions were proposed: (1) it is very difficult, using DNA vaccination, to produce immunogenic E1E2 complexes; and (2) E1 is a very poor immunogen.

Glycans have been shown to influence the immunogenicity of proteins in different ways: through their ability to maintain an appropriate antigenic conformation structurally, through their capacity to shield potential neutralizing epitopes, and through their ability to alter the proteic susceptibility of proteins to proteolytic digestion [52]. Fournillier et al investigated whether removal of defined N-linked oligosaccharide chains could influence HCV E1 capacity to induce humoral- and or cellular-mediated responses [53]. Eight plasmids were used, encoding E1 protein mutants in which the four N-linked glycosylation sites of the protein were mutated separately or in combination. Vaccination studies in mice revealed that mutation of the fourth glycosylation site (N4) significantly enhanced the anti-E1 humoral response in terms of both seroconversion rates and antibody titers. Interestingly, antibodies induced by the N4-mutant were particularly efficient at recognizing HCV-VLPs, suggesting that they may be more fit at neutralizing native particles. These results show that N-linked glycosylation can limit the antibody response to the HCV E1 protein and revealed a potential vaccine candidate with enhanced immunogenicity. Finally, a more recent study comparing immunization with DNA vaccines expressing either the HCV core alone or the whole structural domain (core-E1-E2) in wild type mice or HCV-transgenic mice demonstrates that a hierarchy of CTL response exists when the proteins were expressed as a polypeptide. The most vigorous responses observed were against E2, followed by core and E1 [54]. Vaccinated HCV transgenic mice developed anti-HCV antibodies and CTL specific to the core. These mice were nonetheless tolerant at the CTL level against E2 despite DNA immunization. DNA vaccines expressing the NS3, NS4, and NS5 nonstructural proteins have been shown to be immunogenic in mice and Buffalo rats. In mice, these nonstructural proteins produced strong cellular immune responses, specific antibody responses, and CD4+ T cell responses with a predominant Th1 phenotype. Specific CD8+ CTL responses were demonstrated for NS3 and NS5 [55]. In Buffalo rats, the immune response to all three nonstructural proteins was increased when a bicistronic plasmid expressing the nonstructural proteins and GM-CSF was used [56].

**Live vector vaccines.** The goal of live recombinant vectored vaccines is to allow for de novo synthesis of vaccine antigens selected for their potential to induce CD8+-mediated immune responses. Progress in the knowledge of the biology
and genomic organization of a growing number of microbial agents as well as in the field of biotechnology has resulted in the production of recombinant vectors (from viral or bacterial origin) engineered to deliver vaccine antigens and allow their endogeneous expression by cells of the immunized host. Among the most widely used viral vectors are poxvirus vectors (derived from highly attenuated vaccinia viruses), defective recombinant adenovirus vectors, alphavirus-derived vectors, and herpes virus vectors. Attenuated bacterial vectors such as tuberculosis Mycobacterium bovis bacillus Camette-Guerin (BCG) and Salmonella typhimurium have also yielded encouraging results.

Two groups have so far reported on the use of recombinant adenoviruses expressing HCV structural proteins (core and/or E1 and/or E2). Bruna-Romero et al vaccinated BALB/c mice with a recombinant adenovirus containing core and E1 genes of HCV in place of the adenoviral E1 gene region [3]. Induction of a strong and long-lasting (up to 100 days) specific CTL response, targeting at least 6 different epitopes, could be demonstrated. The cytotoxic T cell response was H-2d–restricted and mediated by T cells with classic CD4+ CD8+ phenotype. In another study, Makimura et al [57] derived replication-deficient recombinant adenoviruses expressing either one or all of the HCV structural proteins that had been inserted in place of the E1A and E3 adenoviral genes. Unfortunately, analysis on injection of the viruses in mice were limited to the detection of HCV antibodies. These included anticore, anti-E1, and anti-E2 antibodies and were found in 100% of vaccinated mice. Two different injection doses were compared [10^{10} and 10^{9} plaque forming unit (PFU)], and data revealed that the lower dose was safer and more efficient. Remarkably, the repeated injection of the vaccine viruses, which resulted in an increase in anti-HCV titers but also in anti-adenovirus antibodies, did not seem to hamper the efficacy of the vaccines.

Preliminary though quite promising results have been obtained with a NS3-transformed Salmonella vector [58]. Oral immunization with Salmonella typhimurium-harboring plasmid DNA has proven efficacious for the induction of cellular mediated immune responses, probably as the plasmid DNA gets delivered into the numerous antigen-presenting cells that are found in the gut-associated lymphoid tissues. In their study, Wedemeyer et al [58] immunized HLA-A2 transgenic mice with increasing doses [10^{2} to 10^{8} colony forming unit (CFU)] of the recombinant Salmonella typhimurium via oral galvage. Specific CTL could be detected following a single injection. They were long lasting (at least 10 months) and appeared to target, although with different efficiencies, three HLA-A2 epitopes previously described in HCV-infected patients. In addition, vaccinated mice were able to control a challenge applied using a NS3–NS4b-expressing recombinant vaccinia virus. No anti-NS3 antibodies could be detected in this study. This vaccine deserves further investigation in higher animal models.

**Combined vaccine regimens**

So-called prime-boost vaccine strategies aim at potentiating the immune response developed by the vaccinee through the consecutive injection of different
vaccines expressing the same immunogens. The basis of these strategies is that vaccines differing in their nature (e.g., a DNA-based vaccine versus a recombinant protein-based one) are likely to differ in their capacity for “priming” (primary response) or for “boosting” (expansion) antigen-specific responses. In the case of HCV vaccine development, only a few such strategies have been so far reported.

Mouse studies

In a first study, Pancholi et al [59] immunized twice H-2d and H-2b mice with a polycistronic DNA-based vaccine (expressing a core-NS3 polyprotein) and boosted them once with a counterpart recombinant canarypox. Enhanced antibody production (a tenfold increase), was observed compared with the DNA vaccine alone. Substantial potentiation of cellular immune responses, in particular the production of IFN-γ, was observed in the case of E1, E2, NS2, and NS3. The protective impact of these responses is currently being evaluated in chimpanzees.

Contrary to these promising results, a similar strategy combining HCV DNA-vaccines and recombinant Semliki forest viruses (rSFV) expressing the same genes, failed to potentiate the HCV-specific induced immune responses [46,60]. In the case of NS3, HLA-A2.1 transgenic mice were used. Neither the DNA vaccine, nor the rSFV, nor their combination, enhanced the vigor of the specific CTL responses nor the frequency of IFN-γ producing cells. Surprisingly, this study revealed that, independent of the vaccine strategy implemented, only 1 out of 4 HLA-A2 epitopes tested were the target of the induced immune response. Such narrow scope of recognition was not modified when the combined vaccine regimen was implemented. Authors of the study speculated that the lack of boosting effect, in this scenario, probably reflected the fact that DNA vaccines and rSFV vaccines use the same mechanism for antigen presentation, a mechanism that is probably different from that used by canarypox-viruses or adenoviruses. Indeed, Himoudi et al [61] have recently shown that boosting HLA-A2.1 transgenic mice, primed with HCV-DNA vaccine, with recombinant adenoviruses yields a significant enhancement of both HCV-specific CTLs and T-cells producing IFN-γ.

Primate studies

A multicomponent prime-boost HCV vaccine strategy has been recently described that targets structural as well as nonstructural proteins [62]. Two chimpanzees were immunized with plasmids expressing the core, E1, E2, and NS3 antigens followed by booster injections with the corresponding recombinant proteins (derived from a genotype 1b isolate). E1-, E2-, and NS3-specific IFN-γ, IL-2, and IL-4 secreting cell populations as well as specific antibodies and proliferative responses were seen in both vaccines, albeit to different levels. One vaccinee developed an overall less vigorous response although, surprisingly, a high peak of IFN-γ was observed 2 weeks before the challenge. This chimpanzee cleared viremia 8 weeks postchallenge as assessed by sensitive PCR, whereas the other chimpanzee controlled the infection as compared with the non-vaccinated control animal. Although only two vaccinees were used in the study, these
preliminary results are very encouraging and point out to a possible critical role of NS3 specific responses in the control of viremia. It is not clear if responses to all four antigens each played a role in the controlling effect observed and additional experiments are warranted to identify the essential antigens beside NS3. The facts that the recombinant antigens were administered in alum (a Th0 type adjuvant) and that a rather more pronounced Th1 response was observed after vaccination suggest that the DNA-priming played a role in the routing of the responses seen.

**Clinical studies**

A version of the vaccine developed at Chiron is presently in phase I clinical trial to study primarily the safety of the vaccine with the MF59 adjuvant as well as investigation of dose. No results of this initial human trial are available at this time. The candidate HCV E1 therapeutic vaccine tested by Innogenetics (Innogenetics, Ghent, Belgium) in chimpanzees has completed a phase I trial, and preliminary results have been presented recently [63]. This study was a single arm study in which 20 healthy male volunteers were given three doses of vaccine in the deltoid muscle according to a three-dose schedule at weeks 0, 3, and 6. A booster dose was applied at week 26. The vaccine was apparently well tolerated and immunogenic as both proliferation and anti-E1 antibodies were detected in all vaccinees after the booster injection. A T-cell stimulation index greater than 3 at week 8 with at least one of the E1 concentrations used was seen in 18 of 20 subjects and in 19 of 19 of the same subjects at week 28. Typical patients with chronic HCV infection mount no or only very weak T-cell responses to this antigen. This vaccine is currently in phase II clinical trial in chronically infected patients.

**Perspectives**

The development of an HCV vaccine, despite substantial progress, remains in an early stage. Numerous strategies have proven capable in mice, and to some extent in primates, to induce vigorous, long-lasting T-cell–mediated responses. The generation of antibodies capable of inhibiting the binding of the E2 antigen or HCV-LPs to susceptible cells in vitro has been achieved. It is now of key importance to address, with some of these vaccine candidates, crucial issues such as memory of the responses induced and their cross-reactivity. The key experiment, consisting of applying the challenge dose after complete disappearance of the vaccine induced responses and not, as typically done, a few weeks only after the last booster injection, remains to be performed. Although the vaccine-related literature is rich in examples of vectored-based or DNA vaccines capable of conferring protection, the examples so far reported for HCV clearly show that only a systematic, careful examination of each given vector will tell whether an antigen-vector formulation displays the most promising immunogenic properties. As mentioned in the introduction of this article, HCV prophylactic or a therapeutic vaccine will likely be constituted differently in part because the
target populations will be different, and in part because an efficacious therapeutic vaccine will probably need more than the induction of strong, broad, and long-lasting specific responses. It will also need to provide help in the restoration of some of the basic functions of cells from the immune system that appear altered during HCV chronicity. These are difficult problems to solve, but the careful selection of appropriate adjuvants or the use of cytokines, chemokines, or costimulatory molecules may be important.

Finally, it should be considered what level of effectiveness would be acceptable for a vaccine against HCV. Virtually all vaccines presently in use are designed to protect against infection or at least acute disease. Acute HCV infections are often mild or even inapparent. The problem with HCV comes primarily from the persistent infection that often results in clinically significant chronic liver disease. In addition, chronically infected individuals account for virtually all transmissions of HCV. If a prophylactic vaccine cannot therefore be devised that would prevent HCV infection completely, a vaccine that would limit the development of chronic infections would likely still be useful and important from both the medical and public health perspectives. In a similar way, the ideal outcome for a therapeutic vaccine would be to eliminate chronic infections with a relatively few doses of vaccine. Such a vaccine would have a profound impact on HCV globally where antiviral therapies are not practical. A therapeutic vaccine capable of improving the immune response and reducing the viral load could have an effect on the development of chronic liver disease, however. Such a response may also become part of a highly successful antiviral approach combined with drugs or interferons. The challenges to the vaccine developers are great, but the importance of this approach should not be underestimated.

References


[50] Lee SW, Cho JH, Sung YC. Optimal induction of hepatitis C virus envelope-specific immunity


