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Abbreviations: cDNA, complementary DNA; CHO cells, Chinese hamster ovary cells; HBc, HBV core; HBV, hepatitis B virus; HCV, hepatitis C virus; HCVcc, hepatitis C virus in cell culture; HCV-LP, HCV-like particle; HCVpp, hepatitis C virus pseudoparticles; IFN, interferon; mAbs, monoclonal antibodies; MLV, murine leukemia virus; NAbs, neutralizing antibodies; PapMV CP, papaya mosaic virus coat protein; TMD, transmembrane domain; VLP, virus-like particle.

Abstract

Given the global prevalence and long-term complications of chronic hepatitis C virus (HCV) infection, HCV constitutes one of the greatest challenges to human health of this decade. Considerable efforts have focused on the development of new effective treatments, but about three to four million individuals become infected each year, adding to the world reservoir of HCV infection. The development of a prophylactic vaccine against hepatitis C virus has thus become an important medical priority. Only a few vaccine candidates have progressed to the clinical phase, and published data on both the efficacy and safety of these vaccines are limited, due to many scientific, logistic and bioethic challenges. Fortunately, new innovative vaccine formulations, modes of vaccination and delivery technologies have been developed in recent years. Several preclinical trials of virus-like particle (VLP)-based vaccination strategies are currently underway and have already generated very promising results. In this commentary, we consider the current state of prophylactic HCV vaccines, the hurdles to be overcome in the future and the various VLP-based vaccination approaches currently being developed.

Introduction

The hepatitis C virus (HCV) is a major pathogen known to cause chronic liver disease, which may progress to cirrhosis and hepatocellular carcinoma. This virus is thus a leading cause of liver transplantation in industrialized countries.[1] Chronic HCV infection is currently a major public health problem, affecting approximately 3% of the world population.[2,3] Moreover, it has been estimated that more than 350,000 people die from HCV-related liver disease each year, worldwide.[4]

Over the last 10 years, it has been shown that progression to severe disease can be prevented by treatment with a combination of pegylated interferon (IFN)-α and ribavirin. However, the efficacy of this treatment is limited, with a sustained virological response rate of only 55%, and this treatment is poorly tolerated, often impairing quality of life.[5,6] Given these disappointing results, researchers have focused their efforts in recent years on characterizing the multiple steps of the HCV life cycle, with a view to identifying new treatment targets for the development of novel antiviral molecules. The standard treatment has, in some cases (particularly in patients infected with HCV genotype 1), recently been supplemented with the newly approved NS3/4A protease inhibitors, better known as telaprevir and boceprevir.[7] This triple therapy increases sustained response rates, but its use is nonetheless limited by its very high cost, potential drug-drug interactions and substantial side effects.

Despite these considerable therapeutic advances, it is estimated that world reservoir of HCV-infected individuals is increased by three to four million newly infected subjects each year. This increase is not limited to developing countries, as the Center for Disease Control and Prevention (CDC) has estimated that 18,000 new HCV infections occur each year in the USA, corresponding to about one new case every 30 minutes.[8] The development of safe, effective and affordable prophylactic vaccines against HCV has thus become a major medical

priority, providing the best long-term hope for controlling the global epidemic, thereby decreasing the burden on healthcare systems. However, there are many obstacles to the development of vaccines against HCV, including the considerable sequence divergence of the HCV RNA genome[9], leading to the emergence of mutants resistant to the humoral and cellular immune responses to infection,[10–12] and the limited availability of convenient small-animal models other than the chimpanzee, mimicking HCV infection in humans. Conversely, there is encouraging evidence that it might be possible to develop a successful vaccine. A subset of acutely infected individuals (15 to 25%) have been shown to eradicate the virus spontaneously[13] and significant levels of natural immunity to HCV have been reported in studies of the chimpanzee model of HCV infection[14] and in studies of reinfections in intravenous drug users.[15] The pessimistic view has also been moderated by advances in our understanding of the immunological correlates and mechanisms underlying the successful control of viral infection.[16] Many studies have demonstrated that the development of robust, broadly cross-reactive, long-lasting cellular immunity mediated by both $CD4^+$ and $CD8^+$ T cells is associated with the resolution of HCV infection.[17–20] It is also becoming increasingly apparent that such responses alone are not sufficient[21] and that neutralizing antibodies (NAbs) also play an important role in conferring protection and facilitating viral clearance.[22–25] The correlates of protective immunity have not yet been completely elucidated, but it is now widely accepted that for a vaccine formulation to provide effective protection against the various genotypes and quasispecies of HCV, it must incorporate epitopes from HCV structural proteins (core, E1, E2) in their correct three-dimensional conformations, to induce the production of high titers of broad NAbs, together with HCV-specific T-cell epitopes from HCV nonstructural proteins (NS3, NS4, NS5), to elicit strong cellular responses.[26]

The recent development of an efficient system for HCV culture[27] and the potential dangers associated with the use of attenuated HCV particles as a vaccine have led to the

development of modern vaccines with different modes of HCV antigen delivery. In recent years, several different strategies, including the use of recombinant proteins, virus-like particles (VLPs), recombinant nonpathogenic live vectors, and prime-boost approaches, have been investigated for use in prophylactic vaccination against HCV, with various degrees of success.[28–31] Some of these vaccine candidates have made it to the clinical phase, but clinical data remain scarce and there are still many hurdles slowing the development of these approaches. However, several preclinical trials involving VLP-based vaccine candidates are currently underway and have already generated very promising results.

Early vaccination strategies based on the recombinant HCV envelope glycoproteins E1 and E2

Most of the successful prophylactic vaccines against viruses developed to date are effective mostly through the action of NAbs. A prophylactic vaccination strategy based on the use of adjuvanted recombinant E1 and E2 viral envelope proteins was, therefore, initially proposed. This prophylactic vaccine effectively protected immunized chimpanzees against experimental intravenous challenge with the homologous 1a strain.[32,33] Moreover, additional studies in chimpanzees demonstrated that this prophylactic vaccine did not result in sterilizing immunity against experimental challenge with a heterologous 1a strain, instead inhibiting disease progression to chronic, persistent infection.[33] As human disease is almost exclusively associated with chronic, persistent HCV infection rather than with acute infection, these data indicated that a prophylactic vaccine based on the E1-E2 viral envelope proteins might be effective at protecting against HCV-associated disease in humans. Furthermore, vaccinated chimpanzees have been shown to produce high titers of NAbs against HCV genotype 1a that can neutralize the *in vitro* infectivity of HCV pseudoparticles (HCVpp) and HCV grown in

cell culture (HCVcc) containing E1 and E2 envelope proteins derived from various genotypes.[34] These data indicate that this recombinant E1/E2 vaccine can elicit cross-neutralizing antibodies targeting broadly conserved epitopes within the diverse *Hepacivirus* genus, with no apparent cellular immune response. Based on these encouraging data, a dose-ranging phase I trial was initiated to explore the safety and immunogenicity of this recombinant E1-E2 vaccine, which was found to be well tolerated and immunogenic in humans volunteers.[28,35,36]

Many other studies have since demonstrated that the resolution of HCV infection is mediated largely by the effects of NAbs raised against the HCV E1 and E2 envelope proteins, which are exposed on the surface of viral particles.[22,23] A recent meta-analysis of the efficiency of HCV vaccines in chimpanzees also demonstrated that the inclusion of all or part of the HCV envelope glycoproteins in vaccines leads to significantly more protective immune responses than are obtained with vaccines based on nonstructural proteins.[37] In addition, recent reports of human monoclonal antibodies (mAbs) neutralizing genetically diverse HCV isolates and protecting against heterologous HCV quasispecies challenge have validated the concept of the use of neutralizing antibodies to prevent HCV infection.[38–41]

These data are all very encouraging, but there is a major obstacle hindering the further development of this vaccine candidate. The coexpression of the full-length E1 and E2 genes has been shown to result in the synthesis of highly mannosylated immature glycoforms with a transmembrane domain (TMD) anchoring them in intracellular compartments in the form of a large, noncovalently linked heterodimer, making the extraction and purification of these proteins extremely difficult and essentially incompatible with industrial development for vaccination purposes.[42,43] These difficulties have led to the development of alternative strategies based on the use of truncated E1 and/or E2 proteins, which are then secreted. However, such approaches have met with limited success, because the deletion of the TMD of

these proteins has been shown to impair their antigenic and functional properties.[44,45]

Promising innovative virus-like particle (VLP)-based vaccination strategies

In the last few years, new vaccination approaches involving the use of VLP-based vaccines have thus been developed to improve the delivery system for HCV neutralizing antibody- and core-specific T-cell epitopes. This approach is justified by previous reports of convincing demonstrations of the efficacy of VLP-based vaccines, particularly for preventing persistent infection and associated diseases caused by hepatitis B virus (HBV)[46] and human papillomavirus.[47]

The generation of HCV-like particles (HCV-LPs) in insect cells has been described and involves the use of a recombinant baculovirus containing the complementary DNA (cDNA) encoding the HCV structural proteins.[48] These HCV-LPs have been shown to have morphological, biophysical and antigenic properties similar to those of the putative virions,[49] and to be highly immunoreactive when incubated with purified antibodies from the serum of patients infected with various HCV genotypes or with various mAbs recognizing conformational determinants, suggesting that they contain correctly assembled HCV structural proteins.[49,50] Many studies on baboons and mice have suggested that HCV-LPs are potent immunogens for the induction of broad, long-lasting antigen-specific cellular and humoral immune responses.[49,51–53] The efficacy of HCV-LPs has been confirmed in a chimpanzee model, in which protective HCV-specific $CD4^+$ and $CD8^+$ T-cell responses were observed.[54] However, the induction of NAbs by HCV-LPs has yet to be demonstrated in the recently developed HCVpp and HCVcc models.[27,55,56]

Modern VLP technology has been shown to be advantageous for the development of safe and effective vaccines, and VLPs have thus been used as useful platforms for delivering

heterologous virus-derived antigens to the immune system.[57] Recombinant retrovirus-derived VLPs formed by the expression of the retroviral Gag protein alone, which can be pseudotyped with a wide array of full-length heterologous viral envelope proteins, have thus become a versatile and efficient platform for vaccination. It has been shown that HCV E1 and E2 envelope proteins can be pseudotyped onto murine leukemia virus (MLV)-Gag retroviral core particles, to generate chimeric infectious particles (HCVpp) (Figure 1A).[58] These particles, displaying E1 and E2 envelope proteins in the correct conformation and maintaining a preferential tropism for hepatic cells, are commonly used to investigate the early events of HCV infection and for the neutralization assays now widely used worldwide.[55,59] More recently, retroviral (MLV)-Gag particles pseudotyped for HCV envelope proteins have been proposed as a new vaccination platform.[60] When used in a prime-boost strategy with HCV-recombinant viral vectors for priming, retroviral particles pseudotyped with E2 and/or E1 HCV envelope proteins have been shown to induce high titers of anti-E2 and/or anti-E1 antibodies, and of NAbs, in both mice and macaques.[60] The NAbs, which were raised against genotype 1a HCV, cross-neutralized the five other genotypes tested (1b, 2a, 2b, 4 and 5) *in vitro*. The results of all these studies were encouraging, supporting the development of retrovirus-derived VLP-based vaccines, but the multiple-dose regimen required to induce a protective immune response nonetheless represents a major difficulty. Moreover, the use of animal retroviral particles for the development of a prophylactic vaccine for humans will require validation, and the large-scale vaccine manufacturing process may represent a bottleneck for the development of vaccines of this type. An alternative approach, overcoming the difficult production of such retrovirus-derived VLPs, involves the direct injection of DNA plasmids encoding proteins generating retrovirus-derived VLPs (plasma-retroVLPs) into mice.[61] This new genetic vaccination approach has been shown to elicit similar HCV-specific immune responses.

The generation of recombinant VLPs by fusing viral antigens of interest to heterologous viral structural proteins that can self-assemble into VLPs also constitutes a promising approach for the induction of HCV-specific immune responses. In recent years, various strategies involving the HBV core (HBc) protein,[62,63] the small (S) HBV envelope protein[64–68] and the papaya mosaic virus coat protein (PapMV CP),[69] which have been shown to self-assemble into VLPs, have been investigated as prophylactic HCV vaccine candidates, with various degrees of success. Many of the immunization assays conducted with such chimeric particles indicated that relatively weak antibody and T-cell responses to HCV epitopes were elicited, suggesting that the nature of the viral epitopes and the site of fusion are determinants of the process of protein assembly into VLPs and of the conformation and immunogenicity of the antigen. However, very encouraging data have recently been reported for immunization with chimeric HBV-HCV envelope particles (Figure 1B). The HBV S protein has been shown to self-assemble into highly immunogenic, noninfectious and secreted subviral particles, which have been used worldwide as a safe, commercial hepatitis B vaccine since the early 1980s.[46] The use of these particles as carriers of small foreign viral antigenic sequences inserted into the antigenic external hydrophilic loop[64–66,70] or the N- or C-terminus[71,72] of HBV S has also since been reported. However, the capacity of these chimeric proteins to self-assemble into VLPs and the induction of an effective immune response were not systematically demonstrated. In efforts to develop an original strategy for incorporating the entire HCV E1 and E2 proteins into these particles, a new concept in vaccine design was recently developed, based on the use of plasmids encoding chimeric HBV-HCV envelope proteins in which the N-terminal TMD of S is replaced with the TMD of E1 or E2.[67] When produced in stably transduced Chinese hamster ovary (CHO) cells, these chimeric HBV-HCV envelope proteins were efficiently coassembled with the wild-type S protein into subviral secreted particles presenting the full-length E1 and E2 proteins in an appropriate

conformation for formation of the E1-E2 heterodimer.[68] These chimeric particles induced a strong specific antibody response to the HCV and HBV envelope proteins in immunized rabbits. More importantly, rabbit sera containing anti-E1 and/or anti-E2 antibodies elicited by this vaccination strategy had cross-neutralizing properties *in vitro* against HCVpp and HCVcc harboring heterologous HCV envelope proteins derived from strains of genotypes 1a, 1b, 2a and 3, highlighting the importance of including both envelope proteins for an effective vaccination strategy.[68] Moreover, the humoral anti-HBs response induced by these particles was shown to be equivalent to that observed in animals immunized with a commercial HBV vaccine. Further studies are required to increase the broadly neutralizing properties of the NAbs induced by this vaccination strategy, and to investigate this specific HCV-neutralizing response in different animal models, but the encouraging data generated by this study support the development of a bivalent HBV-HCV prophylactic vaccine candidate potentially able to prevent initial infection with either of these two hepatotropic viruses. Such a vaccine would be of considerable value, because the populations at risk of infection with HBV and HCV through exposure to infected blood are essentially the same, in both developed and developing countries.[73] In this context, the induction by HBV-HCV envelope particles of an anti-HBs response equivalent to that induced by commercially available HBV vaccines is also important, as it suggests that bivalent HBV-HCV vaccines could replace existing vaccines against HBV whilst providing the additional benefit of protection against HCV. Another major advantage of this approach is that this vaccine candidate could be produced by the same procedures established for HBV vaccines, reducing the time and cost of its industrial development.

Future Perspectives and Conclusions

As many studies in humans and chimpanzees have shown that the effective immune control of HCV infection is clearly associated with the establishment of vigorous, sustained and broadly directed $CD4^+$ and $CD8^+$ T-cell responses,[17–20] the development of prophylactic vaccination approaches has been largely based on attempts to enhance the cellular arm of the adaptive immune response. In the last few years, many recombinant nonpathogenic live vector-based vaccination strategies have thus emerged. In this perspective, a new T-cell HCV genetic vaccination strategy based on the use of a target immunogen spanning the HCV nonstructural genes NS3-NS5B, a region that contains many well defined $CD4^+$ and $CD8^+$ epitopes, has been developed in the form of recombinant replication-deficient human adenovirus constructs or plasmid DNA.[74] The use of this vaccination approach in a multiple prime-boost regimen has been shown to protect chimpanzees against progression to chronic infection after challenge with a heterologous HCV virus, due to the induction of broadly reactive, sustained and vigorous HCV-specific $CD4^+$ and $CD8^+$ T cell-mediated immunity. Despite the promising results obtained with this strategy, the adenoviral vectors were shown to suffer from the limitation that adenoviral infection is common in humans, and preexisting high-titer anti-vector NAbs may interfere with the immunological potency of such vaccines. Attempts have been made to overcome this obstacle and to evaluate the safety and potency of such vaccines in healthy volunteers. Phase I clinical trials have been initiated to explore prime-boost immunization regimens involving the use of two adenoviral vectors based on rare serotypes expressing the HCV NS3-NS5B gene cassette.[75] This vaccination approach has been shown to be highly immunogenic in humans, with the induction of robust, cross-rective and sustained $CD4^+$ and $CD8^+$ T cell-mediated responses, and has also been shown to be safe and well tolerated. Thus, HCV vaccine formulations comprising prime-boost heterologous

immunization with a recombinant live virus-based vector seem to constitute promising approaches for eliciting strong cellular responses with broader ranges of epitopes, with the aim of clearing HCV infection. As the resolution of HCV infection is frequently associated with the development of robust, cross-reactive and long-lasting cellular immunity and the production of NAbs, it would be very interesting to investigate, in a prime-boost regimen, a combination of the bivalent HBV-HCV prophylactic vaccine eliciting anti-E1 and anti-E2 cross-neutralizing antibodies with a vaccine inducing strong cellular immune responses. The ultimate goal of these investigations would be the induction of highly protective, long-lasting immunity to HCV.

In conclusion, promising results for several types of HCV vaccination in clinical trials, including adjuvanted recombinant envelope E1/E2 proteins and prime/boost immunization regimens involving the use of recombinant replication-deficient human adenoviruses expressing nonstructural genes, suggest that it should be possible to develop vaccines with at least partial efficacy against HCV-induced chronic liver disease. In addition, the recently developed VLP-based strategies have already proved highly promising for the development of HCV vaccine candidates. All these encouraging data suggest that it should be possible to develop a prophylactic hepatitis C vaccine in the near future.

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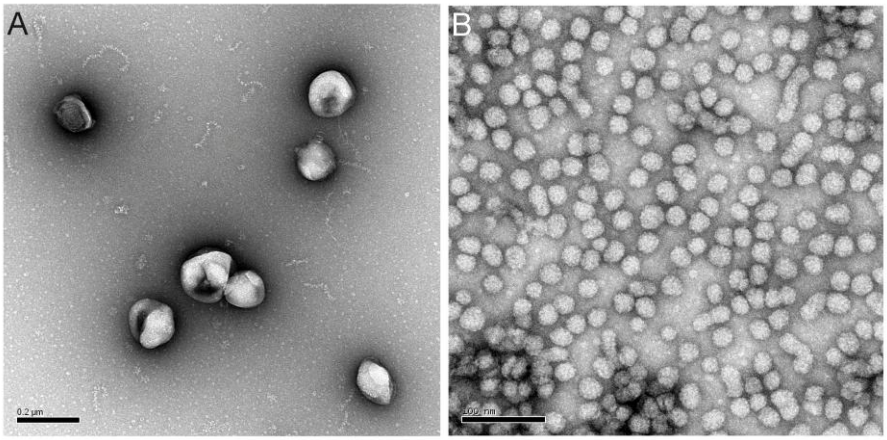

Figure 1. Virus-like particles constitute potential platforms able to carry full-length HCV E1-E2 envelope proteins in an appropriate conformation. A: Murine leukemia virus (MLV) particles formed by the production of the retroviral Gag protein alone can incorporate HCV E1-E2 proteins. B: Hepatitis B virus (HBV) surface protein self-assembles into subviral, noninfectious particles, which can be used as an effective hepatitis B vaccine. Similar particles can be obtained with chimeric proteins formed by the fusion of the HBV S protein with the HCV E1 and/or E2 proteins. Both pseudotyped MLV-like particles and chimeric HBV-HCV subviral envelope particles have been shown to induce antibodies cross-neutralizing various HCV genotypes.