Recent Advances in Development of DNA Vaccines Against *Hepatitis C virus*

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Abstract Hepatitis C is one of the foremost challenging diseases all over the world. No vaccine has been developed, yet against *Hepatitis C virus* (HCV). This is partly due to the high mutation rate in the HCV genome, which generates new genotypes and sub genotypes. A mass of efforts have been devoted for the development of an efficient vaccine against HCV. DNA Vaccines, an emerging field of Vaccinology, grasp strong potential to be the most reliable and efficient mode of vaccination in the future. This technology is under investigation currently. Incredibly diverse approaches have been applied as an endeavor to develop a potent DNA vaccine against HCV. The HCV structural genes and the virus like particles have been attempted and so far the results are quite promising in the Lab animals. As there is no proper animal model for HCV infection except chimpanzees, it is very difficult to articulate whether these vaccines will also be pertinent in humans or not. This review will focus on different approaches being used for the development of DNA vaccines, the major tribulations in designing a DNA vaccine against HCV as well as the future prospects for the improvement of under trials DNA vaccines developed against HCV.

Keywords HCV · DNA vaccines · Virus like particles

Introduction

It is estimated that about 3% of the world population is infected with *Hepatitis C virus* (HCV). Its prevalence is more common in the developing countries of the world. About 1.5 million deaths occur per year due to HCV [4]. Current estimates of global and regional prevalence of HCV are given in Table 1 and Fig. 1. Data suggests that in Middle East Anti-HCV antibodies were found in highest percentage in population (4.7%). This is followed by Africa (3.2%) and Europe (2.3%) [22].

HCV belongs to viral family Flaviviridae [39]. It has positive stranded RNA genome of about 9.5 kb including a large open reading frame (ORF) which encodes a large polyprotein of 3,000 amino acids. There are about 9 genotypes and more than 30 sub-genotypes of HCV throughout the world. The core genotypes of HCV are genotype 1–6, while the subtypes are named as 1a, 1b, 1c and so on [16]. Some of the genotypes like 1a, 1b, 2a, 2b are found all over the world while others like 1b are restricted to specific areas. The genotype 1b more frequently occurs in the Western Europe and Japan, 1a in America, 4 in the Middle East and Central Africa and 1b is found in Turkey [36]. The genotypes of HCV show approximately 30% of diversity in nucleotide sequences of their genomes. In an infected patient, a population of quasi species can be seen due to mutations in the nucleotide sequences of HCV. Particularly the hyper variable region 1 (HVR 1) that is located at the N terminus of E2 protein shows remarkable quasi-species nature [28]. HVR1 region and its quasi-species nature is thought to play an important role in the immune escape of HCV and in the development of chronic infection [9]. The HCV infection can be divided into three main phases, acute, silent and reactivated. Acute phase infection is generally without any symptoms and

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Table 1 Regional prevalence of hepatitis C virus in 2010

Region	Anti-HCV (%)	No. of HCV-infected
Africa	3.2	28,100,000
America	1.5	14,000,000
Asia	2.1	83,000,000
Australia	1.2	400,000
Europe	2.3	17,500,000
Middle East	4.7	16,000,000
Total	2.35	159,000,000

Courtesy by Lavanchy [22]

lasts for six (6) months, after which the infection becomes chronic. While the silent phase generally takes about 10–15 years [1].

A DNA vaccine is most promising technique used so far, capable of producing both humoral and cell mediated immune responses. In several experiments and research works it has been shown that DNA vaccines are much victorious as compared to other types of vaccines, in providing protection against infectious diseases [41], particularly viral diseases [35]. DNA vaccines designed predominantly against different types of cancer, HIV, Influenza, Hepatitis C and other major human diseases are currently under clinical trials [24]. These plasmid encoded vaccines provide a platform for the advancement in the field of vaccinology and molecular biology.

DNA vaccines or the genetic vaccines consist of a bacterial plasmid which contains the gene for the desired antigen, a promoter and a terminator for efficient gene expression [41]. These are constructed by isolating the antigenic gene from the disease causing agent and then cloning those genes into a bacterial plasmid [11]. Upon administration of the vaccine in vivo, the antigen DNA is expressed and processed. The unique element of DNA vaccine is that it elicits a very strong CD4⁺ and CD8⁺ response in vivo. There is very little data available that

explain how the DNA vaccine activates the humoral and cellular immunity. However, it is known that the CpG motifs act as built-in adjuvants in DNA vaccines. It recognizes their unique Toll like Receptor 9 (TLR9) and hence enhances the immunogenicity of the DNA vaccines [21]. It is also reported that the double stranded nature of the DNA vaccine actually exerts the adjuvant effect. It activates the TANK binding kinase 1 (TBK1) dependent innate immune signaling pathway and presents the antigen either directly or indirectly to specialized cells in vivo [29].

Fastidious advantages of DNA vaccine over traditional vaccine is that the DNA vaccine can activate all three forms of the adaptive immunity, named as Helper T Cell response, antibodies and cytotoxic T Cell response. It also avoids cellular transformation and provokes immunity that cannot be induced by wild type infection [32]. It minimizes the risks associated with the possible reversion of the weakened organism to infectious one as in case of attenuated and inactivated vaccines. Problems of limited resources for the large scale implementation are all solved by the DNA vaccines as it is several folds safe and possible to design according to need [41]. Immune responses are diverse and can be biased from T Helper Cell type 1 (Th1) to T Helper Cell type 2 (Th2) [40]. Studies have also shown that continuous administration of DNA vaccine does not elicit any anti-DNA antibody [46].

DNA vaccines that particularly elicit T cell response in the host has been shown to be more desirable in viral infectious diseases. The plasmid vector carry antigenic gene when it is delivered into host cell where it is transcribed and mRNA is formed. The mRNA is translated into the protein product and is usually cleaved by the proteosomes of the host cell. The resulting peptides are then represented on the cell surface by Major Histo-Compatibility Complex 1 (MHC 1) as antigen/MHC1 complex, which binds CD8⁺ T cells. This induces cell mediated response, which is particularly required in case of virus

Fig. 1 Epidemiology of hepatitis C virus around the globe in 2010. Courtesy by Lavanchy [22]

antigens. DNA vaccines are more efficient than other vaccines because their antigenic epitopes more closely resemble the native viral proteins. In case of traditional vaccines, the proteins are altered either during the manufacturing process or during the delivery of vaccines. Moreover, after delivery into the body, the proteins are more often modified by the cellular environment and hence fail to elicit a strong immune response. DNA vaccines have one more advantage over other vaccines as in case of DNA Vaccines multiple antigens can be administered at a single dose by the construction of a polycistronic vaccine which encodes antigens of different origins [15].

In contrast to conventional vaccines that are aimed to induce humoral responses, DNA vaccines primarily activate cytotoxic T lymphocytes, involved in the clearance of the virus. Also there are a lot of risk factors associated with the introduction of xenogenous substances, as it is a common procedure in conventional vaccines. All these possibilities are limited and avoided in the preparation of DNA vaccines [34]. DNA vaccines are also capable to act against antigenic shift and antigenic drift variants. This is an additional benefit and can be useful for HCV vaccination [26].

The use of viral vectors for the administration of DNA vaccines can be beneficial as well as harmful for the host. In case the gene of interest is integrated in the host genome, it can lead to mutagenesis and can induce carcinogenesis, by either activating an oncogene or inactivating tumor suppressor gene. It can also render a functional gene into a non functional gene. The DNA vaccine may also create autoimmune disorder and immunologic tolerance, as in majority of cases, especially in eukaryotes where the product is very much less [25].

Why is it Difficult to Manufacture HCV Vaccine?

The genome variability in HCV is very high, and that is the biggest hurdle towards stepping vaccination attempts against it. The HCV virus is constantly mutating itself and this is an important part of its survival strategy. HCV produces 1,012 new viruses per day inside human body and the HCV NS5B RNA polymerase, which lacks proof-reading activities, introduces about 1 error in every 10,000 bases made [10]. As a comparison, human DNA polymerase, whose function is to copy DNA at cell division, introduces about 1 error in 10,000,000,000 bases made and other error-preventing systems even reduces this rate further. Doing some combinatorial arithmetic, assuming an even distribution of mutations in the HCV genome, an infected person can potentially carry a virus with every single nucleotide mutation and 10% of all double mutations at any one time [14]. This variability makes it difficult to

design drugs/vaccines against HCV. Since the drugs are targeted against specialized viruses, reducing viral loads. Evolution selects resistant quasi-species of the virus towards which the drug becomes ineffective.

HCV RNA encodes a large poly protein of about 3,010–3,033 amino acid residues [42] that is post translationally processed by both viral and host proteases. The proteins closer to the N terminal of the polyprotein are called the structural proteins, while those closer to the carboxy terminal are called non structural proteins [19]. Two non-translated regions are also present known as 5' NTR and 3' NTR, present at the 5' and 3' terminals respectively [15]. Structural region of HCV consists of four proteins, namely: Core protein (C), two envelop proteins (E1 and E2) and a small protein p7, and these proteins are thought to play a crucial role in the formation of viral particle. The non structural proteins of HCV consist of NS2, NS3, NS4 and NS5. The genome order of HCV has been determined to be 5'-C-E1-E2-p7-NS2(p23)-NS3-NS4A-NS4B-NS5A-NS5B-3' [6]. All these genes have been tested for the development of DNA vaccines, but the non structural genes particularly the two envelop proteins and the core protein remained the center of attention for the researchers since a long time. Recently the non structural genes are also utilized both individually and in combination with the structural genes for the development of a competent vaccine against HCV.

Secondly, HCV is unable to evoke sterile immunity; the re-infection from the same strain or different genotype can occur following first infection [17]. For the production of an effective vaccine against HCV, it is necessary that the vaccine should induce long lasting, high titer and cross reactive antibodies and should also be capable of evoking T cell mediated immunity. DNA vaccines are the prime candidates that can have all the properties of a good vaccine against HCV. Such DNA Vaccines can be designed that will induce T helper response and it will in turn induce both Th1 and Th2 responses against HCV [5].

DNA vaccines against HCV are the current research focus in many well known virology and vaccinology research centers. As the main hurdle in the development of a DNA Vaccine is the high mutation rate of HCV, researchers are exploiting the conserved regions in the genome of HCV for development of vaccine. HCV core is commonly exploited as it is the most conserved region in the HCV genome [18]. The envelope glycoproteins are quite variable regions but in the start these regions were not considered as potent for the development of vaccine; however, these regions are also engineered today along with E2 HVR1. In one study, E2 glycoproteins and HVR1 were used. The chimpanzee model used to inoculate the vaccine, showed both cytotoxic T cell response and produced antibodies to E2 and HVR1 [20]. HCV nonstructural

proteins are also under investigation for the development of DNA vaccines because of their significance in the viral life cycle and for being conserved areas in most of the genotypes of HCV. Previous literature indicates that DNA vaccines are very potent in the development of both T cell and B cell immunity against HCV. Researchers are paying attention on proper delivery system for transformation of cells for the development of a more effective DNA vaccine [8].

This review will focus on the recent advances in the development of DNA vaccines against HCV, the identification of a potent adjuvant and its effect on the efficacy of currently developed DNA vaccines and the development of a suitable delivery system for the efficient expression of the vaccine genes.

DNA Vaccines Against HCV

Development of Alphaviral Particle

Lin et al. [27] reported that broad CD4⁺ and CD8⁺ T cell responses can be induced by the vaccination of Th1-Adjuvanted polypeptide. It is followed by alphaviral particles expressing the gpE1, gpE2, NS3, NS4 and NS5. By such vaccination cross reacting antibodies are also produced against HCV. In various experiments, recombinant HCV polypeptides were combined with various Th1 type adjuvants and replication defective alphavirus particles which encoded HCV proteins in different prime/boost modalities. The defective alphaviral particles which encoded gpE1/gpE2 heterodimer and NS345 elicited very strong CD8⁺ T cell response but the CD4⁺ response was very weak in BALB/C mice. When gpE1/gpE2 heterodimer was adjuvanted with MF59 with CpG oligonucleotide, the CD4⁺ response was enhanced but no CD8⁺ T cell response was observed in the subjects. When recombinant NS345 was used, the CD4⁺ T helper cell response was very high but ultimately there was no CD8⁺ T cell response even the prep was adjuvanted with Icosamatrix CpG. Both CD4⁺ and CD8⁺ responses were observed when first the prep was adjuvanted with Th1 type protein and then boosted with chimeric effective alphavirus particles that contained the HCV genes. This prime/boost prep resulted in the induction of anti-E1E2 antibodies that were cross reactive against a number of other genotypes [27].

Although such type of DNA vaccine showed promising results in BALB/c mice but whether it will work in humans or not, is still a question. Moreover, as the alphaviral particles are replication defective, so the antigens may not be represented sufficiently in humans.

The main problem associated with the alpha viral particles prime/boost vaccine is its inability to replicate in the

host cells. In some studies, viral vectors able to replicate inside the host cell and which expressed HCV genes were used. Viral capsid, E1, E2, p7, NS2 and NS3 genes of genotype 1b of HCV were engineered in vaccinia virus vector system and a recombinant vaccinia virus (rVV) was tailored that expressed all these genes. Chimpanzee models were immunized with the rVV. After the challenge, all the animals resolved the infection. Control animals, who received only parental rVV, developed chronic infection. The immunized animals were, then subjected to other genotypes of HCV, the vaccine gave cross protection against several genotypes, particularly against genotype 4 and 5 [45]. This suggests that replicating Vaccinia virus may be a potent candidate for development of DNA vaccine against HCV.

In another study, recombinant virus like particles were developed as an attempt for the production of DNA vaccines against HCV. HCcAg 120-VLPs were designed that contained hepatitis C surface antigen with three other peptides that were A1, A2 and A3. The VLPs were injected to BALB/c mice alone and in combination with different adjuvants. HCcAg 120-VLPs elicited both antibodies against the surface antigen and a highly specific T cell response. Moreover, the formulation also induced delayed type of hypersensitivity response and induced Th1 cytokines in the mice. When the formulation was used with an adjuvant like Montanide ISA888, it resulted in a remarkable control of the virus titer. After immunization with VLPs-adjuvant formulation, the mice were subjected to recombinant vaccinia virus which expressed HCV core proteins. The immunized mice showed marked increase in specific IFN- γ in their spleen cells [33]. This indicates that recombinant VLPs may also be fruitful in the formulation of a novel genetic vaccine against HCV.

Development of Polytopic Vaccines

To be potent enough against HCV, a vaccine should be designed such that the CD8⁺ T cell response against the virus is rigorous and highly specific. With such an idea, a polytope vaccine was designed that is comprised of one H-2Dd (E2 protein) and two HLA-A 0201 (E1 and core). The format of the minigene was E2E1C for optimum epitope expression and presentation. The DNA sequence was cloned in pcDNA3.1 vector system. Immune enhancer elements, Universal T helper epitope (PADRE), endoplasmic reticulum signal sequence and HBsAg genes were also cloned in the formulation both separately and in combination. Expression and presentation of the epitope was enhanced with the use of enhancer sequences and endoplasmic reticulum signal sequences in cell lines [3]. The vaccine is not yet tested in experimental animals and/or in humans but it is thought to be valuable in the

development of DNA vaccine against HCV. Wen B et al. also studied the immunogenicity of E2 gene of HCV. The E2 gene alone can induce the T Cell response against the virus but the response is not sustained. In contrast, when Globular Domain of Human Adiponectin gene was fused with E2, the response was greater. The fusion gene was expressed in mice and the mice showed a higher T Cell response against the virus [44]. This study can contribute a lot towards the development of a DNA vaccine against HCV.

The HCV core protein has immune-suppressive properties and suppresses the action of antigen presenting cells and T cells. It has been shown that core alone can lead to sustained suppression of CD3⁺ and CD4⁺ T cells. So it is not a good candidate for vaccine development alone. However, if some other appropriate immunogen is linked with the core gene; it can induce both humoral and cellular response [7]. The core protein of HCV has been shown to suppress the immune responses when expressed in liver cells in terms of inhibition of granzyme B and cytokines production. However, if the dendritic cells are transfected with the core gene, it leads to the expression of regulatory T cells. This is responsible for the persistent infection. However, if conjugated with NS3, the response may be biased to viral clearance by production of both effector and regulatory T cells [23, 30].

Development of Bacillus Calmete Gurein Based Vaccine

In the most recent years *Bacillus Calmete Gurein* (BCG) is being used as a vector system in the field of DNA vaccination. It is already in use as an effective delivery system in a number of bacterial, viral and other parasitic infections. Recently, it was subjugated in the HCV epitope delivery experiments, as a sound delivery system for HCV vaccines is still a major research problem. Being relatively inexpensive, heat stable and capable to carry large amount of DNA, BCG is presently in use in many countries. Incidences of complications are also very low, and itself it can induce T-cell responses. Therefore, it is a good candidate for the development of vaccines against HCV.

Two plasmids pDE22-CtEm and pDE22-CS1 were incorporated into BCG Tokyo strain. These plasmids were carrying genes for the Core, E2 and CTL epitopes that have effectively expressed the HCV multi-epitope antigen CtEm and HLA-A2.1. Transgenic mice were immunized with the recombinant BCG strains rBCG-CtEm. High levels of antibodies specifically targeted towards the multi-epitopes of HVR1 were produced in the serum of transgenic mice. However, the humoral immune response was good only in rBCG-CtEm group. The antibody level was only detectable in the initial inoculation and increased many folds upon

booster injection. In vitro, HCV epitope specific cellular immunity was also induced in the mice [43]. The results from BCG based vaccines were very satisfactory in Lab animals, however, it is still to be verified whether these vaccines are going to be productive in future or not.

Development of NS Genes Based DNA Vaccines

In contrast to the structural genes of HCV that are significant for the production of strong immune response, some studies revealed that non structural genes are also chief candidates for the development of DNA vaccines. HCV genes, NS2 and NS5a, that play a role in viral pathogenesis and resistance to interferon treatment, were cloned in a eukaryotic expression vector, pcDNA 3.1(+), which encoded the full size NS5a protein. Mice were immunized three times with the construct that induced a broad spectrum cellular immunity directed against NS5a protein. After immunization with the recombinant plasmid, the mice were subjected to viral challenge. The mice produced antiviral cytokines IFN γ and IL2. A novel adjuvant immunomodulator, Immunomax was used with the construct which showed high activity in the genetic immunization. The T cell response was also enhanced by using the adjuvant-construct mixture [31]. The results were good enough and suggest further work on this system. As these genes are involved in pathogenesis and interferon resistance, the implementation of such type of vaccine can be problematic.

A strong T cell response against NS3 protein of HCV has been reported in a variety of studies in acutely infected and chronically infected HCV patients [12]. Also the NS3 protein can induce both class I and class II restricted T cell responses, which are directly related to the clearance of the virus [38]. The T cell induced granzyme B (GrB) production is also directly related to the viral clearance. It has been shown that NS3 can increase the production of both CD4⁺ GrB production and CD8⁺ GrB production. However, if the NS3 is primed with the core gene the GrB production can be increased to three or four folds. Hence, the NS3 is a potent candidate for the development of prophylactic and/or therapeutic DNA vaccine that will be able to induce a strong T cell response and will ultimately lead to the clearance of virus [13].

If the pathways, by which the regulatory T cell response may be reversed or blocked, are known, it will help in designing a potent immunotherapy and/or a DNA vaccine against HCV.

Development of Glycoproteins Based DNA vaccines

The glycoproteins of HCV, E1 and E2 are the major targets of antibodies because they play a significant role in viral

entrance to the cell [17]. Previous studies on immunity against HCV infection suggests that T-Cell responses induced in patients are strong and multi-specific and are involved in viral clearance from the body. Very little is known about the antibody response during infection, partly because of the unavailability of proper cell culture system for the virus [15]. But these problems are overcome by the retrovirus-HCV pseudoparticles (HCVpp) and JFH1 strain based cell culture system (HCVcc). In different studies, E1, E2 and p7 genes, individually and in combination have been used as potential vaccine candidates. Rodents and guinea pigs were immunized with all these versions of the genes and their efficacy was tested. Oil/water adjuvant MF59-0 was used for mouse immunization, while MF59-0 and MF75 were used for the immunization of guinea pigs. E1E2p7, E1E2 and E2 elicited strong humoral response against the genotype from which previously the genes were isolated and cloned, and produced strong neutralizing antibodies in HCVpp (HCV pseudo-particles) and HCVcc (Cell culture grown HCV) based systems. E2 alone was also sufficient to induce antibodies in HCVpp and HCVcc systems. When the experiments were performed in human subjects, the antibody titer reduced 3 folds [47] because of the presence of the lipoproteins in the human sera. This indicated that E2 and E1E2 can induce polyclonal humoral response that also have cross reacting activities and hence may be used in future for the formulation of a therapeutic or prophylactic vaccine against HCV.

Vaccines that Have Entered Phase 2 Clinical Trials

Only two DNA vaccines, CIGB-230 and ChronVac-C have entered clinical trials. CIGB-230 is constructed from plasmid pIDKE2 that express HCV structural antigens and HCV core protein. The HCV core protein is sought to play a role as a potent immunogen. This vaccine induces a very strong antibody based and T cell based immunity against HCV in Lab animals. In the phase 1 of clinical trial, the patients immunized, produced antibodies to the viral pseudo particles and 46% of the patients cleared the infection. One third of the patients also produced cellular immunity against the infection. The liver histology of the patients was improved and the risk of fibrosis was reduced. In the phase of 2 trials, the vaccine is used in combination with IFN and antiviral agents like Ribavirin for the treatment of HCV [37]. CIGB-230 vaccine in combination with interferon's and ribavirin is currently suggested in infection in some parts of the world.

The other vaccine ChronVac-C is codon optimized NS3 and NS4a genes which are expressed under the control of CMV immediate early promoter. Till now, the reported data shows that the vaccine has no side effects, and T cell response becomes evident after the second dose of the

vaccine. However, the complete data has not yet been shown to the public and the trials are going on [2].

Conclusion

Today our knowledge regarding HCV has become broadened due to the extensive research done on kinetics and pathogenesis of HCV. Above studies show that a lot of research has been done for the development of DNA vaccine against HCV, this is because of the significance, efficacy and thorough understanding in the field of DNA vaccines. No doubt the development of virus like particles is a great gift of molecular virology and is very promising in the development of DNA vaccines in future. Still a lot of research is required in designing a vaccine that should be polycistronic and represent multiple epitopes of HCV. The vaccine should be effective against multiple though not all genotypes of HCV and should also elicit both T cell immunity and B cell immunity; Particularly, the cellular arm of immunity that is responsible for viral control in humans and also in clearing HCV from body during HCV infection. Moreover, a novel immuno-competent transgenic animal model should be engineered to help us identifying the ineffective vaccines before administering in human body. In the light of current research, we can conclude that in near future, a DNA vaccine that would be effective against all the genotypes of HCV will be available in the market.

References

1. Ali SMA, Lixin X, Ruofeng Y, Xiaokai S, Xiangrui L. Construction of DNA vaccines encoding *Eimeria acervulina* cSZ-2 with chicken IL-2 and IFN- γ and their induced immunity against poultry Coccidiosis. *Res Vet Sci*. 2011;90:72–7.
2. Alvarez LL, Carrera SD. Commentary advances in DNA immunization against *hepatitis C virus* infection. Opportunities and challenges. *Human Vaccines*. 2009;5:568–70.
3. Arashkia A, Roohvand F, Memarnejadian A, Aghasadeghi MR, Rafati S. Construction of HCV-polytope vaccine candidates harboring immune-enhancer sequences and primary evaluation of their immunogenicity in BALB/c mice. *Virus Genes*. 2009;40:44–52.
4. Arribillaga L, de Cerio AL, Sarobe P, Casares N, Gorraiz M, Vales A, Bruna-Romero O, Borrás-Cuesta F, Paranhos-Baccala G, Prieto J, Ruiz J, Lasarte JJ. Vaccination with an adenoviral vector encoding *hepatitis C virus* (HCV) NS3 protein protects against infection with HCV-recombinant *vaccinia virus*. *Vaccine*. 2002;21:202–10.
5. Britton RA, Lin DC, Grossman AD. Characterization of a prokaryotic SMC protein involved in chromosome partitioning. *Genes Dev*. 1998;12:1254–9.
6. Bukh J, Forns X, Emerson SU, Purcell RH. Studies of *hepatitis C virus* in Chimpanzees and their importance for vaccine development. *Intervirology*. 2001;44:132–42.

7. Cao J, Chen Z, Rena Y, Luo Y, Cao M, Luc W, Zhao P, Qia Z. Oral immunization with attenuated *Salmonella* carrying a co-expression plasmid encoding the core and E2 proteins of *hepatitis C virus* capable of inducing cellular immune responses and neutralizing antibodies in mice. *Vaccine*. 2011;29:3714–23.
8. Chen W. Investigation of Functionalized carbon nanotubes as a delivery system for enhanced gene expression with implications in developing DNA vaccines for *Hepatitis C Virus*. University of Saskatchewan Library Electronic Theses & Dissertations. 2008; 17–21.
9. Choi MJ, Kim JH, Maibach HI. Topical DNA vaccination with DNA/lipid based complex. *Curr Drug Deliv*. 2006;3:37–45.
10. Choo QL, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coi D, Medina-Selby A, Barr PJ, Weiner AJ, Bradley DW, Kuo G, Houghton M. Genetic organization and diversity of the *hepatitis C virus*. *Proc Natl Acad Sci*. 1991;88:2451–5.
11. Coban C, Koyama S, Takeshita F, Akira S, Ishii KJ. Molecular and cellular mechanisms of DNA vaccines. *Hum Vaccin*. 2008;4: 453–6.
12. Cooper S, Erickson AL, Adams EJ, Kansopon J, Weiner AJ, Chien DY, et al. Analysis of a successful immune response against *hepatitis C virus*. *Immunity*. 1999;10:439–49.
13. Deepa KK, Li W, Kumar B, Tyrrell DL, Agrawal B. HCV-core and NS3 antigens play disparate role in inducing regulatory or effector T cells, in vivo: Implications for viral persistence or clearance. *Vaccine*. 2010;28:2104–14.
14. Deleersnyder V, Pillel A, Wychowski C, Blight K, Xu J, Hahn YS, Rice CM, Dubuisson J. Formation of native *hepatitis C virus* glycoprotein complexes. *J Virol*. 1997;71:697–704.
15. Donnelly JJ, Ulmer JB. DNA vaccines for viral diseases. *Braz J Med Biol Res*. 1999;32:215–22.
16. Dusheiko G, Schmilovitz-Weiss H, Brown D, McOmish F, Yap PL, Sherlock S, McIntyre N, Simmonds P. *Hepatitis C virus* genotypes: An investigation of type-specific differences in geographic origin and disease. *Hepatology*. 1994;19:13–8.
17. Feltquate DM, Heaney S, Webster RG, Robinson HL. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J Immunol*. 1997;158:2278–84.
18. Greenwood B, Jeffrey C, Strickland G. Jenner Vaccine Foundation NEWS (November 2010). Oxford, 4.
19. Han JH, Shyamala V, Richman KH, Brauer MJ, Irvine B, Urdea MS, Tekamp-Olsen P, Kuo G, Choo QL, Houghton M. Characterization of the terminal regions of *hepatitis C virus* RNA: Identification of conserved sequences in the 5' untranslated region and poly(A) tails at the 3' end. *Proc Natl Acad Sci USA*. 1991;88:1711–5.
20. Higashi Y, Kakumu S, Yoshioka K, Wakita T, Mizokami M, Ohba K, Ito Y, Ishikawa T, Takayanagi M, Nagai Y. Dynamics of genome change in the E2/NS1 region of *Hepatitis C Virus*, in vivo. *Virology*. 1993;197:659–68.
21. Ishii KJ, Coban C, Kato H, Takahashi K, Torii Y, Takeshita F, Ludwig H, Sutter G, Suzuki K, Hemmi H, Sato S, Yamamoto M, Uematsu S, Kawai T, Takeuchi O, Akira S. A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. *Nat Immunol*. 2006;7:40–8.
22. Kato N, Hijikata M, Nakagawa M, Ootsuyama Y, Muraiso K, Ohkoshi S, Shimotohno K. Molecular structure of the Japanese *hepatitis C* viral genome. *FEBS Lett*. 1991;280:325–8.
23. Krishnadas DK, Li W, Kumar R, Tyrrell DLJ, Agrawal B. In vitro activation and differentiation of naïve CD4⁺ and CD8⁺ T cells into HCV core- and NS3-specific armed effector cells: a new role for CD4⁺ T cells. *Cell Immunol*. 2009;259:141–9.
24. Lang KA, Yan J, Draghia AR, Khan A, Weiner DB. Strong HCV NS3 and NS4A specific cellular immune responses induced in mice and *Rhesus macaques* by a novel HCV genotype 1a/1b consensus DNA vaccine. *Vaccine*. 2008;26:6225–31.
25. Lavanchy D. Evolving epidemiology of *hepatitis C virus*. *Clin Microbiol Infect*. 2011;17:107–15.
26. Lenz O. Resistance to HCV protease inhibitors. 2nd Nordic Conference of HIV and Hepatitis Drug Resistance and Treatment Strategies, (2008). Uppsala, Sweden.
27. Lin Y, Kwon T, Polo J, Zhu YF, Coates S, Crawford K, Dong C, Wininger M, Hall J, Selby M, Coit D, Medina-Selby A, McCain C, Ng P, Drane D, Chien D, Han J, Vajdy M, Houghton M. Induction of broad CD4⁺ and CD8⁺ T-cell responses and cross-neutralizing antibodies against *Hepatitis C Virus* by vaccination with Th1-adjuvanted polypeptides followed by defective alpha-viral particles expressing envelope glycoproteins gpE1 and gpE2 and nonstructural proteins 3, 4, and 5. *J Virol*. 2008;82:7492–503.
28. Liu MA. DNA vaccines: a review. *J Intern Med*. 2003;253: 402–10.
29. Liu MA. DNA vaccines: an historical perspective and view to the future. *Immunol Rev*. 2011;239:62–84.
30. Lukens JR, Cruise MW, Lassen MG, Hahn YS. Blockade of PD-1/B7–H1 interaction restores effector CD8⁺ T cell responses in a *hepatitis C virus* core-murine model. *J Immunol*. 2008;180: 4875–84.
31. Masalova OV, Lesnova EI, Grabovetskii VV, Smirnov OA, Ulanov TI, Burkov AN, Ivanov AV, Zaberezhnyi AD, Ataulakhov RI, Kushch AA. DNA Immunization with a plasmid carrying the gene of *hepatitis C virus* PROTEIN 5A (NS5A) induces an effective cellular immune response. *Mol Biol (Mosk)*. 2009;44:275–83.
32. Nabel GJ, Kaslow DC, Ulmer JB, Liu MA. DNA vaccines. In: Levine MM, et al., editors. *New generation vaccines*. 4th ed. New York: Informa Healthcare USA, Inc; 2010. p. 386–95.
33. Nelson AR, Poutou J, Alvarez L, Lajonchere Guerra I, Aguilera Y, Musacchio A, Rodriguez A, Guir JC, Falcon V, Obregon JCA, Soria Y, Torres D, Linares M, Perez A, Grillo JM, Carrera SD. Recombinant in vitro assembled *hepatitis C virus* core particles induce strong specific immunity enhanced by formulation with an oil-based adjuvant. *Biol Res*. 2009;42:41–56.
34. Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, Perelson AJ. *Hepatitis C* viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science*. 1998;282: 103–7.
35. Ole Olesen (2008). *Vaccines for humans*. Publication Office European Commission, Directorate health unit F3: infectious disease. 19–28.
36. Saito S, Kato N, Hijikata M, Gunji T, Itabashi M, Kondo M, Tanaka K, Shimotohno K. Comparison of hypervariable regions (HVR1 and HVR2) in positive- and negative-stranded *hepatitis C virus* RNA in cancerous and non-cancerous liver tissue, peripheral blood mononuclear cells and serum from a patient with hepatocellular carcinoma. *Int J Cancer*. 1996;67:199–203.
37. Sallberg M, Diepolder H, Jung MC, Frelin L, Mathiesen I, Fons M, et al. Antiviral effects of therapeutic vaccination with naked DNA delivery by in vivo electroporation in patients with chronic *hepatitis C*. *Hepatology*. 2008;48:1022–3.
38. Shoukry NH, Sidney J, Sette A, Walker CM. Conserved hierarchy of helper T cell responses in a chimpanzee during primary and secondary *hepatitis C virus* infections. *J Immunol*. 2004;172: 483–92.
39. Simmonds P. Variability of *hepatitis C virus*. [Review]. *Hepatology*. 1995;21:570–83.
40. Smith HA. Regulation and review of DNA vaccine products. *Dev Biol (Basel)*. 2000;104:57–62.
41. Ulmer JB, Warren B, Liu MA. Gene-based vaccines: recent technical and clinical advances. *Trends Mol Med*. 2006;12: 216–22.
42. Van Doorn LJ. Molecular biology of the *hepatitis C virus*. *J Med Virol*. 1994;43:345–56.

43. Wei SH, Yin W, An QX, Lei YF, Hu XB, Yang J, Lu X, Zhang H, Xu ZK. A novel *hepatitis C virus* vaccine approach using recombinant *Bacillus Calmette-Guerin* expressing multi-epitope antigen. *Arch Virol*. 2008;153:1021–9.
44. Wen B, Deng Y, Tan WJ, Ying X, Gao JM, Ruan L. Construction and evaluation of *hepatitis C virus* (HCV) DNA vaccine containing E2-gAD fusion gene. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi*. 2010;24:53–5.
45. Youn JW, Hu YW, Tricoche N, Pfahler W, Shata MT, Dreux M, Cosset FL, Folgori A, Lee DH, Brotman B, Prince AM. Evidence for protection against chronic *Hepatitis C Virus* infection in Chimpanzees by immunization with replicating recombinant *vaccinia virus*. *J Virol*. 2008;82:10896–905.
46. Zambon M. Epidemiology and pathogenesis of influenza. *J Antimicrob Chemother*. 1999;44:3–9.
47. Zania S, Coates S, Evans MJ, Wininger M, Crawford K, Dong C, Fong Y, Chien D, Abrignani S, Balfe P, Rice CM, McKeating JA, Houghton M. *Hepatitis C virus* envelope glycoprotein immunization of rodents elicits cross-reactive neutralizing antibodies. *Vaccine*. 2007;25:7773–84.