Hepatitis: A Global Health Concern

Chapter 6

Plant-Made Vaccines to Combat Hepatitis; A Global Perspective

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1. Introduction

Viral hepatitis is the most common causative agent of inflammation and damage of the liver. Viral hepatitis is frequently caused by hepatitis A, B or C viruses. In general, Hepatitis A Virus (HAV) causes the least amount of liver damage. This virus is found worldwide, but predominates in regions of the world with poor sanitation. Infection lasts for a relatively short period of time, and the virus is not fatal. Hepatitis B, on the other hand, infects approximately one- third of the human population. Hepatitis B Virus (HBV) can lead to a number of pathologies, the most severe being hepatocellular carcinoma (HCC), or liver cancer. HBV often causes chronic liver disease, with two-thirds of those afflicted being unaware that they carry the virus. Hepatitis C Virus also leads to chronic liver disease and sometimes cancer. All three of these viruses come from diverse virus families and have no relation to each other, save the fact that they preferentially infect hepatocytes and cause hepatitis. All are transmitted through different means, and possess widely different replication and translational strategies. An efficaceous vaccine exists for Hepatitis A and B, but not for hepatitis C (Centers for Disease Control and Prevention https://www.cdc.gov/hepatitis/abc/index.htm).

Over the past twenty years, plants have been used as production platforms to develop vaccines and other pharmaceutical proteins. Initially generated to help the rural poor in non-Industrialized countries, plant-made vaccines are easy to upscale, inexpensive to produce and can be stored at ambient temperatures, all major stumbling blocks for conventional vaccine

production in remote parts of the world. In addition to this, plants possess post-translational modifications that resemble their mammalian counterparts. Plants cannot act as hosts for human pathogens such as cytomegalovirus, which makes them safer production platforms for vaccines, monoclonal antibodies, and other pharmaceuticals. Plant-made vaccine constructs generally lack intellectual property constraints and are provided freedom-to -operate status, both major advantages for helping the poor. The fact that vaccines can be expressed in plants that are routinely eaten, such as tomatoes, maize or rice, and can be delivered with simple partial purification steps, further facilitates their use for developing countries. While transgenic plants were the first form of vaccine expression platform used, more recently, plant viruses have been increasingly employed as vaccine expression systems. Plant viruses can produce enormous amounts of protein within a short period of time and are not constricted by public perception concerns regarding genetically modified organisms.

The following chapter provides an overview of the current state of the art of development of plant-made vaccines against viral hepatitis. The chapter begins with a discussion of strategies used and successes associated with the design of plant-made vaccines to combat Hepatitis B Virus. The chapter then addresses an innovative approach which is currently underway and that could result in the first effective plant-made vaccine against HCV. Finally, the chapter presents strategies used to create a vaccine in plants to target HAV infection. The chapter concludes with a summary of the progress that has been made in the field of viral hepatitis and the importance of this research in providing affordable medicines to the poor of developing countries.

2. Plant Made Vaccines Against Hepatitis B Virus

2.1. Worldwide spread of hepatitis b virus infection:

The causative infectious agent of serum Hepatitis worldwide is the Hepatitis B Virus (HBV). It is a serious disease with considerable morbidity and mortality, and is widespread with over 2 billion people infected with HBV, of which nearly 350 million are chronic carriers of the virus. HBV is a blood-borne virus causing infections in humans and chimpanzees [1] and is also shed into other body secretions including saliva, semen and vaginal fluids. The major mode of virus spread is through sexual intercourse, blood transfusion and the use infected needles or equipment. Every year, over 1 million people die [2,3,4] due to HBV disease with a high incidence of hepatocellular carcinoma (liver cancer) and cirrhosis along with other symptoms of virus-induced morbidity.

2.2. Urgency of need for vaccine against HBV:

Due to the pandemic nature of this virus, there is great demand for generating the HBV vaccine [5], compounded with the inability to grow the virus in in vitro cell cultures or

laboratory animals.

2.3. Biochemistry and Molecular Biology of HBV:

Deciphering the molecular biology of the HBV genome and virus structure is essential towards developing vaccine strategies for prophylaxis and therapy of HBV infection. HBV is a small icosahedral DNA virus with a diameter of 30 nm, belonging to the family Hepadnaviridae. It is enveloped inside a lipid coat in which are anchored 3 surface proteins. The genome of the virus is composed of a circular DNA molecule that is partly double-stranded with its long strand having a length of 3.2 kb and the short strand varying in length between 1.7-2.8 kb [6,7]. The virus gene, env, has 3 start codons, coding for the three surface proteins, namely, small (S; HBsAg), composed of 226 residues, medium (M; preS2-HBsAg) composed of 281 residues and large (L; preS1-preS2-HBsAg) containing 389 residues [6, 8]. Thus, all 3 proteins share the same open reading frame and a common S domain. The virion surface is composed of the major S antigen (HBsAg) and the minor M and L forms of the HBsAg. All three forms of the antigens exist in glycosylated forms, although the S and L antigens exist also in non-glycosylated forms [4]. The S-antigen by itself assembles into stable, immunogenic VLPs which exhibit an octahedral symmetry [9,10]. VLPs or Virus-Like Particles are self-assembled structures composed of viral antigens that mimic the architecture of the complete viruses, but are not infective due to lack of the viral genome. The virus replicates by reverse transcription [11]. During the course of viral replication inside the cells, an excess of surface antigen is generated, forming spheres or rods of diameter 22 nm composed of structured HBV lipids and proteins that form the HBsAg particles, and which rampantly circulate in the blood of carriers. The primary target of HBV is the liver and therefore it is hepatotropic.

2.4. History of development of HBV vaccine and the need for more innovative vaccination systems:

The primary immunogenic determinants against HBV infection are purified, non-infectious HBsAg particles making them ideal for mass vaccination protocols [4,5,12]. Multisubunit HBsAg particles obtained and purified from the blood plasma of individuals chronically infected with HBV was first used in 1981 as a HBV vaccine [13,14]. However, since this was not amenable for mass vaccination, genetic engineering was resorted to in order to generate subunit HBsAg vaccines. Initially, attempts to construct recombinant clones of HBsAg in bacterial systems were unsuccessful. Subsequently, the yeast system and mammalian cell cultures (particularly, the Chinese Hamster Ovary (CHO) cells) proved favorably conducive for generation of robust vaccines against HBV [4]. A typical immunization course of three inoculations protects a majority of infants (95%) and adults (90%) [15]. However, despite its successful vaccination efficiency, it is not suitable for mass vaccinations especially in underdeveloped and developing countries due to its high price, the need for highly qualified

personnel to administer it and lack of 100% success in generating immunity against HBV despite the administration of this vaccine. Also, there is insufficient medical infrastructure for cold chain distribution and increasing demands for a stable, robust HBV vaccine in poor countries, especially due to mutated HBV strains that emerge in response to increased selection pressure occurring because of vaccination [16]. Also forming a barrier towards effective vaccination is the emergence of immune senescence which often occurs in adults [17]. Moreover, it is not possible to express M and L antigens in bacteria and even in yeasts as they exhibit a necessity for specific conditions to enable their expression [18]. The commercial vaccine is a HBsAg subunit vaccine generated in yeast and administered by injection as a three-dose series along with adjuvants. However, it is not thermo stable, losing its immunogenicity upon freeze-thawing [19,20] or upon storage for a week at 45C [21]. Therefore, there is a compelling need to generate more cost-effective, safe and simply administered vaccines, one of which could be a mucosal vaccine.

2.5. Emerging concept of plant-derived vaccines against HBV and advantages of plant-based vaccines:

The advent of transgenic plants producing HBV antigenic proteins provides for the development of oral / mucosal vaccines against HBV [22, 23]. The advantage of a plant-delivered HBV vaccine is that the plant cell walls ensure efficient shielding of the enclosed antigen after ingestion and in the acidic environment of the stomach. Thus, the antigen packaged in the plant cell reaches the intestines whereupon it is displayed to the mucosal immune system that in turn stimulates immune reaction against the HBV antigen. The most attractive prospect of such oral / edible vaccines is their low cost [23], dispensing with the need to isolate and purify the immunogen, simplified storage and safe needle-free immunization, their medical safety with no contaminants such as human viruses / mycoplasmas/prions / other human pathogens. Also, plant-derived HBsAg could be used as raw or dried foodstuffs. Purified vaccine antigens can be encapsulated, dispensing with the need for cold chain systems for transport and delivery. Furthermore, oral vaccines overcome the inherent difficulties in parenteral administration systems. For over the past 15 years, a lot of research resources have been invested in the development of plant-based oral vaccines against HBV [24].

3. Immunogenicity of the HBsAgs Produced in Transgenic Plants

The first formulated HBV vaccine in transgenic tobacco plants emerged in 1992 [25] wherein VLPs of ~22 nm were generated, which were found to recognize monoclonal antibodies to HBsAg. Following this, in 1995, this plant extract was injected into mice whereupon a specific and robust immune response equivalent to that of the yeast-derived subunit vaccine was generated [26]. This established the feasibility of using transgenic plants for production of HBV antigens and their use in vaccines against HBV. On the other hand, use of edible

plants for generating oral vaccines seems more practical. The first edible HBV immunogens were synthesized in transgenic lupine and lettuce plants. Subsequently, transgenic potato synthesizing HBsAg was constructed and was shown to elicit robust immune response in mice and volunteers [27,28], wherein the latter showed increased serum antibodies to HBsAg when fed with raw transgenic potato after primary immunization with a subunit HBV vaccine. Also, the neutralizing activity of HBV vaccine composed of the recombinant surface antigen HBsAg was augmented by the addition of preS1 and preS2 regions of the HBsAg [29,30,31]. However, transgenic potato was not ideal because 100 g of raw potato had to be ingested in a single dose, and any attempts to enhance digestibility by boiling the potato led to 25-fold reduction in immunogenicity [27,28]. On the other hand, transgenic maize proved to overcome these hurdles providing higher expression levels of the HBsAg in a more palatable and easily digestible composition [32], while exhibiting stronger immunogenic reactions at both systemic and mucosal sites [32,33] in addition to being an inexpensive, thermo stable formulation [33]. In this context, it is important to mention that the HBsAg particles contain 30% lipids by mass [34, 35, 36] and these lipids are essential to enable the self-assembly of the HBsAg into VLPs in addition to influencing its immunogenicity [34,37,38]. The processing procedures that the plants expressing the VLPs are subjected to, can influence this lipid association. In the transgenic maize expression system, delipidation is a preferred step to generating a highly thermostable vaccine [Hayden et al., 2012 [33]. Nevertheless, this very delipidation step can impact the immunogenicity of the maize-derived HBsAg [32].

Of late, the HBV S and M antigens were expressed in transgenic carrots [39] whose advantage lies in the lack of need for thermal processing before consumption; however, this proved to be unsuccessful in raising robust anti-HBV response when tested in mice. The M antigen was found to be more susceptible to protease cleavage compared to the S antigen. Subsequently, transgenic rice plants were engineered to express the SS1 protein composed of the HBsAg from which 3 amino acids from the C-terminus was deleted, followed by fusion with the preS1 sequence [40]. VLPs characteristic of HBsAg was found to be expressed in the seeds of these transgenic rice plants. Total protein extracts from these seeds along with Freund's adjuvant when injected thrice into mice intraperitoneally proved successful in generating preS1 and HBsAg-specific antibodies, showing that the recombinant SS1 protein is a good candidate for an alternate version of the HBV vaccine. Another protein composed of 9 immunogenic epitopes of the HIV-1 Env and Gag proteins (TBI) was fused to HBsAg at its N-terminus and expressed in transgenic tomato plants, thus generating a combined edible vaccine against both HIV-1 and HBV [41].

Also, transgenic tobacco and lettuce plants were generated, stably expressing M and L HBV antigens which assembled into VLPs and analogous aggregates [42].

3.1. Augmenting the immunogenicity of plant vaccines based on HBsAg

HBsAg is able to be absorbed on mucosal surfaces by virtue of resembling effective mucosal pathogens which are multimeric and organized as supramolecular entities [43]. The immunogenicity of HBsAg edible vaccines is augmented by the use of mucosal adjuvants such as Cholera Toxin B subunit, heat-labile enterotoxin B subunit and saponins [15,44]. Also worthy of mention are adjuvants derived from flavonoids and plant oils. At present, aluminium hydroxide is the only licensed adjuvant being medically administered, the main caveat of which is that it weakly stimulates cell-mediated immune reaction. Tomatin, a saponin sourced from edible tomato fruits [45] is an important candidate as a good, non-toxic mucosal adjuvant by virtue of being plant-sourced and an essential food crop. Therefore, the use of transgenic tomatoes is doubly advantageous in providing both the HBsAg and adjuvant functions [46, 47].

3.2 Augmenting the yield of HBsAg in plants

A major problem in HBsAg production in transgenic plants is its low yield which consequently results in diminished immune response. This necessitates the augmentation of HBsAg yield in the transgenic plants [48,49]. One way is to direct the HBsAg produced in the plant into specialized cellular compartments such as endoplasmic reticulum [50] or vacuoles [51,52] using appropriate signal peptides that help in protecting the HBsAg from proteolysis.

Thus, the preS2-S M antigen was targeted to the ER lumen as sigER-preS2-HBsAg-HDEL in transgenic carrot plants and this resulted in enhanced yield of the M antigen [53].

3.3. Transplastomic expression of HBsAg

Also, elevating the gene dose by using the transplastomic chloroplast system is another alternative. The greatest expression of transgenes, up to over 70% of the total soluble protein has been reported for antigen expression in chloroplasts [54,55]. Plastids, by virtue of being polyploid and repetitive, increases the HBsAg copy number to 1000-10,000 copies per cell even when a single copy of HBsAg is introduced into the plastid DNA. This was demonstrated in transplastomic tobacco plants wherein the yield of the foreign protein was up to 1-25% of the total level of soluble protein [56] that far exceeds the protein amount produced in transgenic plants. Moreover, plastids exhibit maternal inheritance and are therefore absent in the pollen. This ensures the environmental safety of the transplastomic plants compared to the transgenic plants due to elimination of the unbridled spread of the transgene to the other plants in the field. Of late, the transplastomic HBsAg has been demonstrated in tomatoes, potatoes and carrots [23,57,58].

3.4. Effectiveness of Transient Expression Systems

Plant virus expression vectors are increasingly being used as transient expression systems wherein the recombinant proteins exhibit rapid, enhanced expression several fold

higher than that of the stable transgenic plants. For instance, MagniCON HBsAg viral vectors when infiltrated into tobacco leaves, produced up to 300 ug of HBsAg per g of fresh leaf weight on day 10 following infection [48] and was subsequently shown to be immunogenic in mice. Thus, transient expression system forms an attractive alternative due to its containment, the short interval between transformation and subsequent expression (unlike the protracted stable transformation [59,60]) environmental safety, rapid manufacturing cycles, diminished cost of genetic engineering and economically viable vaccine production. Transient expression is achieved through transfer DNA delivery using Agro bacterium tumefaciens (also called Agro-infiltration) and / or virus-based replication systems. For HBV, transient expression has been carried out in N. benthamiana, and both stable as well as transient expression has been achieved in potato, tobacco, tomato, carrot, lettuce and Arabidopsis.

3.5. Genetic engineering parameters affecting the yield of plant-based HBsAg vaccines:

Augmenting vaccine production in plant systems involves developing efficient genetic engineering strategies such as strong constitutive promoters, promoters expressed only in selective tissues, as well as those induced by varying the environmental conditions, optimum codon usage, alternative polyadenylation signals, the use of leader sequences to augment translation efficiency, various vector systems and signal peptides for targeting the vaccine antigen into specific organelles such as endoplasmic reticulum, vacuoles or chloroplasts. The most popular constitutive promoter under use is the Cauliflower Mosaic Virus 35S promoter with enhancer [61-63]. Tissue-specific promoters such as fruit-specific promoters [64,65], patatin promoter for expression in potato tubers [62,66] and globulin promoter for expression in maize seeds [33,67] have been reported. The ubiquitin promoter and ethylene forming enzyme promoter have also been used in transgenic tobacco [68]. The former has been shown to be more efficient in greenhouse-grown plantlets whereas the latter has been better expressed in in vitro-cultured plantlets. In potato, maximal expression of HBsAg was found to be driven by a construct containing the CaMV 35S promoter with dual enhancers, the tobacco etch virus 50 – UTR and the 30 region from the soybean vegetative storage protein gene [62]. Also, using tuber-specific promoter further increased the expression levels. Expression in tomato fruit and banana leaves [65] were reportedly 0.5 ug/g dry weight and 38 ng/g fresh weight respectively. Another alternative is to express HBsAg in grains such as maize and rice, which ensure long stability of the expressed recombinant HBsAg with low water content [69]. An expression level of 0.51% of the total soluble protein (80 ug/g fresh weight) was reported for HBsAg in maize seeds using barley alpha amylase signal sequence fused to the HBsAg gene driven by the 3x globulin1 promoter [33,67]. HBsAg derived from transgenic tobacco plants was found to be immunologically, physically and antigenically similar to the HBsAg raised in yeast and in human serum [25]. Transgenic soybean has been used to generate up to 74 ug/g fresh weight of HBsAg [70]. On the other hand, suspension culture from transgenic

tobacco is used to secrete HBsAg into the culture medium, which when supplemented with salicylic acid or jasmonic acid, augmented the amount secreted to 180 ug/L medium [65,70]. Tomatoes are very attractive candidates for development of oral vaccine [11]. Using the maize system, a robust immune response of up to 4632 mIU of maximum titer was generated by oral administration and injection [33,67,71]. This appears promising towards development of thermostable formulations.

3.6. Caveats in the expression of HBsAg in plants towards generating a plant-based vaccine

The major inherent problem with transgenic plants is that the target DNA containing the HBsAg is randomly inserted into the plant genome during Agrobacterium-mediated transformation. Therefore, even with the same vector construction, there are differences in the expression levels of the HBsAg due to position-specific effects. Also, the expression levels of the HBsAg vary widely depending on the plant species, tissue types and culture conditions. Another caveat to contend with in raising plant-based HBsAg vaccine is its low yield and inability to be produced at a competitive price compared to the presently used yeast-derived vaccine. The slow rate of growth of plant cells compared to that of yeast is another factor to contend with. Plus, there are difficulties in administering bulky plant matter to individuals, diminishing long-term responses, individual variations in immune response and anomalies in defining antigen dose [72]. Besides, the M-HBsAg and L-HBsAg are much less studied than S-HBsAg [1].

Furthermore, a very important factor in administering transgenically-derived HBsAg vaccines is to consider the public's acceptance of genetically modified crops in particular, that of plant-derived oral vaccines. Also, before acceptance of plant-based edible vaccines, human risk assessment and environmental risk evaluations have to be conducted. All plant-based vaccines should be generated in accordance with good manufacturing practices, just as for conventional vaccines.

4. Conclusions

Tomatoes, bananas, lettuce and carrots are advantageous in being food crops that dispense with thermal processing prior to consumption and therefore are ideal candidates for HBsAg production. Also, freeze-drying storage roots and fruits of the transgenic plants followed by grinding into powder forms or pelleting/encapsulating seem to be very practically feasible and provide effectual means of vaccine delivery. Recombinant HBsAg could also be preserved in plant seeds and grains such as transgenic rice, maize and other cereals. Freeze-dried formulations dispense with the need for complicated antigen preparation while affording compact simple packaging, enhanced storage stability, ease of handling and regulated administration which is ideal for mass vaccination regimens in developing countries [73-76].

On the whole, a combination of injection as well as oral HBV vaccines, seem to be the most promising regimen to provide protection against HBV infection. Plant viral vectors, transgenic and transplastomic plants producing HBV S, M and L antigens (that assemble into VLPs) could provide a viable, economic alternative compared to the conventional yeast or mammalian cell systems that are currently in use. Plant-based oral "triple" anti-HBV vaccine mass-producing the S, M and L forms of the HBV surface antigens can become the novel third-generation vaccine effective for mass vaccination programmes. Tri-component vaccines are demonstrably more effective compared to sole S-HBsAg vaccines and are increasingly being used in vaccination programme [17,77,78].

5. Plant Made Vaccines to Combat Hepatitis C Infection

5.1. Physical features of Hepatitis C and current vaccine development

5.1.1. Hepatitis C discovery and obstacles in vaccine development

In 1975, it was recognized that most cases of hepatitis acquired from blood transfusion were not caused by either hepatitis A or B [79]. This mysterious virus is invisible under the microscope due to its instability during the staining process. It wasn't until 1989, this "non-A, non-B hepatitis" was identified as the new hepatitis C virus (HCV) by Choo *et al.* through genome sequencing instead of classical virus purification. HCV is one of the main causative agents of hepatitis in the world prevailing in up to 170 million of the population, mostly in Africa and in Eastern Mediterranean regions [80]. Egypt has the highest incidence of HCV (type 4) in the world, estimated at 14.7% [81] of the country's population. HCV subverts the host immune mechanism to establish persistent / chronic infection with very low rate of spontaneous recovery [82]. The infection is characterized by serious complications such as liver dysfunction, hepatocellular carcinoma and liver cirrhosis [83-85].

HCV is not only troublesome due to its invisibility under the microscope but its highly variable surface protein, envelope protein-2 (E2) also make the eradication of the virus difficult [86]. HCV has a unique hyper variable region (HVR-1) spanning 27 amino acids on the N-terminus of E2. The error prone viral RNA polymerase contributes to HVR-1 which culminates in a diverse population of quasi-species [87]. Due to the high genetic variability of the virus, HCV has been classified into seven major sub-genotypes [88]. These HCV sub-genotypes contribute to different disease symptoms and have different responses to direct antiviral agent. As a result, around 3% of population worldwide are chronically infected with HCV and this infection can progress into liver fibrosis, cirrhosis and hepatocellular carcinoma [89].

This situation underscores the dire need to develop effective vaccinations against HCV and combat the persistent and progressive state of the disease. Tenacious, robust Cytotoxic

T Lymphocyte (CTL) response specific to HCV epitopes is the central immune mechanism characterizing the body's defense against HCV disease [90,91,92].

5.1.2. Structural and physiological properties of Hepatitis C virus

HCV is an icosahedral virus belonging to the *Flavivirus* group [93, 94]. The structure of HCV is composed of Core protein, E1 and E2 **(Figure 1).** The Core protein assembles into the icosahedral structure of HCV which is 30-60 nm in diameter **(Table 1).** The density of HCV varies between 1.12g/cm^3 to 1.18g/cm^3 and has a sedimentation coefficient ranging from 180S-215S [95]. The differences in the measurement of densities and sedimentation coefficient are due to variations in the structures of viruses for distinct subgenotypes and association with different host lipoproteins [96]. The E1 and E2 glycoproteins are type I transmembrane proteins that include N-terminus ectodomain and C-terminus transmembrane domain anchored on the surface of HCV capsid [97]. The E1 and E2 are released from the polyprotein precursor after cleavage by the host endoplasmic reticulum (ER) signal peptide peptidase and viral protease (NS2-3 and NS3-4A) [98]. After being released, E1 and E2 are transferred to the endoplasmic reticulum (ER) lumen to be modified with N-linked glycosylation [97]. Different glycosylation patterns on E1 or E2 can result in different isoforms with varying molecular weight [97]. The glycosylation of E1 and E2 have been shown to be important in the viral stability, immunogenicity and biological functions [97]. This suggested that the glycosylation on E1 and E2 have essential roles in the association with host molecules such as human CD81, scavenger-1, low-density lipoprotein (LDL) and Claudin-1 receptor for viral entry [99]. Therefore, glycosylated envelope proteins are important targets for neutralizing antibodies which is the key for vaccine development [100].

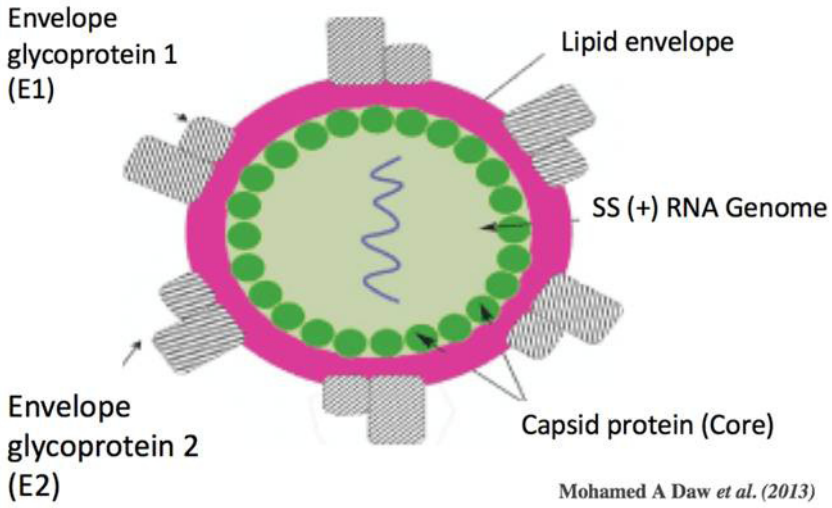

Figure 1: HCV structure. The surface of HCV is composed of Core protein, E1 and E2. HCV genome is a plus-sensed single stranded RNA.

Table1: Molecular weight of HCV structure proteins: Core, E1 and E2

Protiens	Molecular weight (kDa)
Core	23 (Precursor) 21 (Mature)
E1	30-35 based on different glycosylation pattern
E2	70-72 based on different glycosylation pattern

5.1.3. Genome structure and organization

HCV genome is a linear plus-sense, single stranded RNA with 9.6 kilo-bases (kb) in length (6) **(Figure 2).** It has an internal ribosome entry site (IRES) at 5' end and a Poly-C tail at 3' end [87]. The sequence of HCV has one open reading frame (ORFs) encoding 10 proteins. IRES-mediated translation of these ORFs leads to the formation of a polyprotein that is processed into 10 viral proteins via both cellular signal peptidase and viral proteases. At the 5' terminus of the genome, it encodes three structure proteins Core, E1 and E2. The rest of ORFs encode non-structural proteins including proteases and RNA dependent RNA polymerase involved in viral replication, infection and assembly.

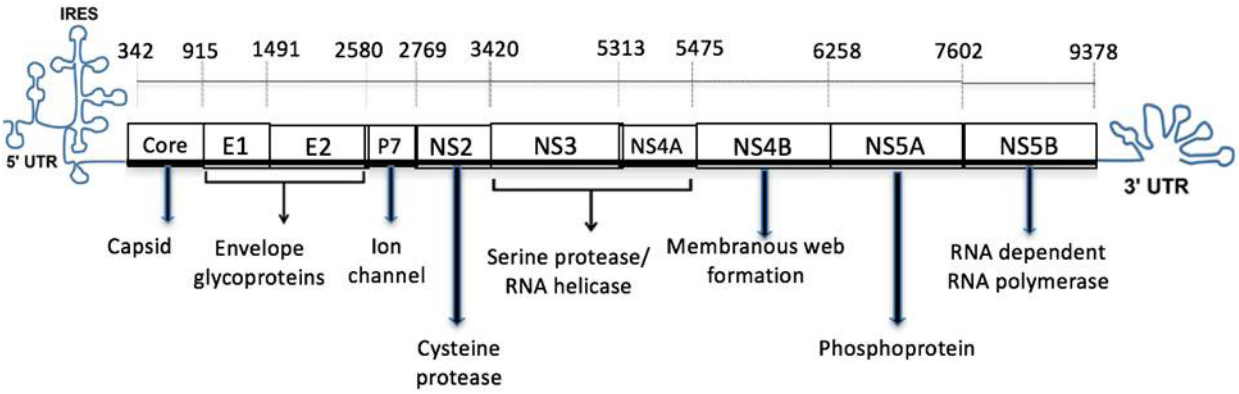

Figure 2: HCV genome and polyprotein. HCV genome has 10 open reading frames and it is 9.6 kb in length. The translation of HCV genome depends on IRES and the polyprotein is processed by cellular and viral proteases [85,87].

6. Accumulation of HCV Mutants During the Course of HCV Infection and Development of Vaccine Against the HVR Region

HCV infection is complicated by variants resulting out of high mutation rate during the virus replication cycle. In particular, the instability of neutralizing epitopes of the virus and the subsequent selection of immune evasion mutants [101,102] lead to unsuccessful immune response to HCV infection. It is because of this reason that HCV has been classified into specific genotypes and subtypes spread over different geographic regions and risk groups [103] HCV mutations are principally concentrated in the hypervariable regions (HVRs) and in particular, the region encompassing the N-terminal segment of the envelope 2 glycoprotein (E2) called HVR1 has been demonstrated to be the crucial HCV neutralization domain [101,104,105]. The HVR1 region also contains B and T cell epitopes and is therefore the major target for developing HCV vaccine [106,107].

6.1. Epitope Mapping of Antibodies Directed Against HVR-1 In E2.

From epitope mapping of HCV E2, it was found that most neutralizing antibodies are targeted against HVR-1 through HVR-3 corresponding to the regions from N-terminus to C-terminus of HVR respectively [92, 108]. HVR-1 is the most highly immunogenic sequence among these 3 HVR sub-regions, but it is also the most variable sequence in HCV and allows the virus to escape host immune response. However, the variability in HVR-1 is not unlimited because this region also contains highly conserved residues for the interaction with SR-BI and CD-81 receptors [108]. Therefore, HVR-1 can be broadly subdivided into the highly variable N-terminal domain, which might serve as immunological bait and the less variable C-terminal domain which serves important functions in associating with host molecules [103,104].

6.2. Broadly Neutralizing Antibody Targeted Against Conserved Region of E2

Broadly neutralizing antibodies targeting the conserved region of E2, including the conserved region of HVR-1 have been shown to be effective in blocking the HCV entry in HuH-7 cells, but fail to protect the HCV infection in chimpanzee model [109]. This suggested that even though, these regions are relatively conserved in HCV E2, there are multiple mutational hotspots that might still render the host immune response ineffective [109]. The effectiveness of broadly neutralizing antibodies in blocking the viral entry in HuH-7 cell culture can be explained by the fact that homogenous HuH-7 cells lack the immune response induced by T-Cells and B-cells. The sequence evolution of HVR-1 is driven by selection under the immune pressure which is shown by relatively stable HVR-1 in immune-deficient patients [108]. One way of resolving the problem of HVR-1 heterogeneity is to immunize patients with combinations of various HVRs derived from different quasi-species of HCV [108]. This gives rise to the idea of generating a polyvalent vaccine against HCV.

6.3. Development of HCV Pseudo-Particles System Using MLV/ HIV

HCV pseudo-particles (HCVpps) are produced from the inoculation system which allows the incorporation of functional HCV envelope proteins into other retroviral capsid such as those of the murine leukemia virus (MLV) or human immunodeficiency virus (HIV) [110]. HCVpp is a more convenient system compared to the wild-type virus in allowing the investigation of cell entry and antibodies neutralization mediated by HCV envelope proteins. The main reason for using HCVpps is that they allow the quantification of a single inoculation event since they lack the viral genome to replicate in the host cells. The quantification of HCVpps inoculation is achieved by luciferase reporter gene expression which is in close association with retroviral packaging construct. HCVpps-luciferase system is important for assessing the functionality of HCV Core-E1-E2 clones and neutralization efficacy of specific antibodies.

Two lentiviral packaging constructs, MLV firefly luciferase reporter plasmid (pNCA-C-

XN.MuLV.FLuc) and HIV renilla luciferase reporter plasmid (pNL4-3.Luc.R-E-) were tested to compare which vector gives a better formation of functional HCVpps and a more precise quantification of HCVpps entry **(Figure 3)** [111]. Each lentiviral vector encodes functional Gag (Core protein), Pol (reverse transcriptase) genes but site directed mutagenesis renders the original lentiviral Env (envelope protein) and Vpr (nuclear import protein) non-functional. The assembly of the pseudo-virus and viral entry depends solely on HCV envelope proteins.

HCVpps were produced by co-transfecting either MLV luciferase reporter plasmid or HIV luciferase reporter plasmid and a HCV expression vector (pcDNA-3.1-Core-E1-E2) into human embryonic kidney cells (HEK cells) (Figure 4). Transfected HEK cells secrete HCVpps into *Dulbecco's Modified Eagle's medium* (DMEM) which can be used for the inoculation of target human hepatocellular carcinoma cells (Huh-7 cells). Expression of HCV structural proteins in either MLV or HIV capsid can be monitored by luciferase reporter assay conducted on Huh-7 cells. Upon inoculation of Huh-7 cells, the luciferase gene is reverse transcribed by lentiviral reverse transcriptase and integrated into the genome of the target cells. Successful transduction of the luciferase gene can be used as an indicator for successful viral entry of HuH-7 cells. In theory, only Huh-7 cells inoculation with functional HCVpps will express luciferase in a detectable level.

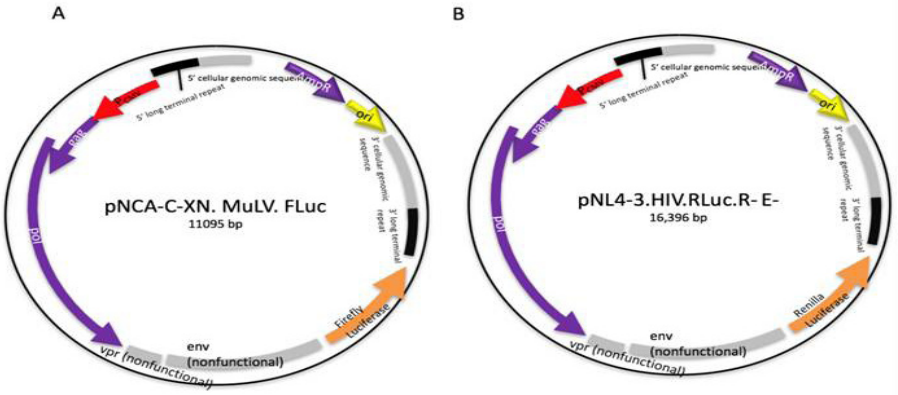

Figure 3: (A) Plasmid map of MLVFirefly luciferase reporter vector (pNCA-C-XN.MuLV.FLuc). (B) Plasmid map of HIVRenilla luciferase reporter plasmid (pNL4-3. RLuc.R-E-).

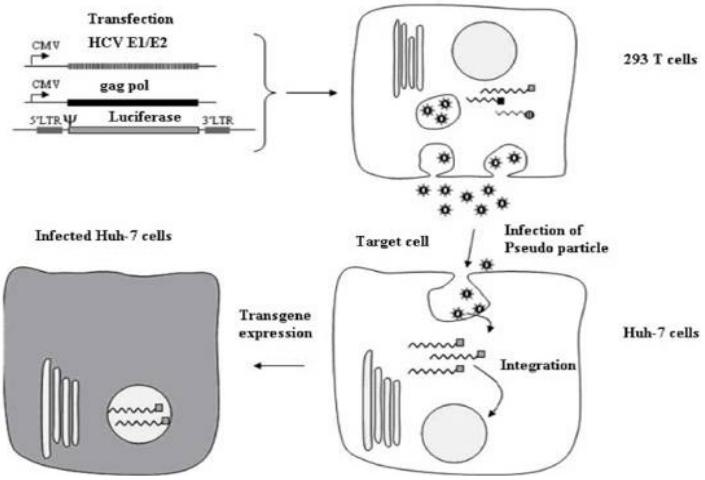

Figure 4: Mechanism of HCVpps production in HEK cells and inoculation of Huh-7 cells. Luciferase gene is packaged in HCVpps and it is reverse transcribed and integrated into Huh-7 host genome [111a].

7. Plant-Derived Vaccines Against HCV

Viral nucleocapsids or Virus-like Particles (VLPs) are self-assembled viral capsid proteins with repetitive structure, bereft of the viral genome and are highly immunogenic while dispensing with concerns regarding biosafety of killed or live attenuated whole virus. Additionally, VLPs derived from plant viruses can be used to express immunogenic epitopes of human viruses that stimulate strong T- and B-cell response and overcome any risk of contamination from bacteria, viruses and prions commonly associated with animal-derived vaccines. This is demonstrated by the immunogenicity of VLPs derived from Papaya Mosaic Virus capsid protein carrying at the C-terminus Hepatitis C Virus E2 epitope wherein humoral and cell-mediated immune responses were shown against both the PapMV-CP and the fused HCV E2 epitope [112]. However, the strength of immunogenicity was dependent on antigen organization and multimerization. The immune response was strong, prolonged and suggestive of a balanced antibody, memory B cell and Th1/Th2 response. Such self-assembly of the VLPs was also discovered when the PapMV CP was expressed in E. coli [110].

Tremblay et al. [110] have shown that the expression of PapMV capsid proteins (CP) in the absence of viral genome generates helical VLPs that are similar to that of the wild-type virus. This will provide the potential of mass producing VLPs expressing HCV epitope in a cost-effective way which can be used for neutralizing antibodies production. The proposed methods involve cloning the cDNA encoding PapMV capsid protein into PET 29c E-coli expression vector. The CP of PapMV has a unique XbaI site just downstream of the start codon that can be used to clone HCV E2 epitope. This PMVCP-E2 construct could then be transformed into E-coli to generate PapMV empty viral capsid with HCV E2 epitope expressing on the surface. These to PMVCP-E2 particles will then be purified and injected into rabbit for neutralizing antibodies production. The antibodies generated will be tested for their neutralization efficacy in blocking the entry of HCVpps in HuH-7 cell.

Another plant virus namely, the Cucumber Mosaic Virus (CMV) has also been used to generate a plant-based HCV vaccine [113,114]. The advantage of CMV is that it is ubiquitous and has a very wide host range including several edible crops such as celery, lettuce, cucumber, tomato, carrot, pepper, and banana as well as the non-edible tobacco. R9-CMV, a CMV construct engineered to express a HCV-derived 27 amino acid synthetic peptide was shown to be stable under acidic stomach and physiological conditions while being able to stimulate strong humoral immune reaction in rabbits that were fed with lettuce plants infected with R9-CMV. This indicated that stable and edible HCV vaccines can be produced in bioreactors for enabling commercial production. The R9 mimotope, a synthetic peptide derived from over 200 hypervariable region 1 (HVR-1) sequences of the E2 envelope protein of HCV was fused to the CMV sequence to generate chimeric CMV, which was subsequently shown to elicit humoral immune response against neutralizing HCV epitopes in rabbits fed with pepper, tobacco and

tomato infected with the chimeric R9-CMV [115-118]. Similarly, using R9-CMV, in vitro anti-HCV cytotoxic T cell responses (CTL) have been shown in patients with chronic hepatitis [118]. This chimeric CMV, when infected and expressed in lettuce was estimated to contain 1.6 mg of the HCV epitope per 100 g of fresh tissue which was shown to be immunologically effective under experimental conditions. This shows great promise in assembling the chimeric CMV into nanoparticles which can be used as an edible, oral vaccine against HCV.

Further to the initial success with R9-CMV chimera, wherein the R9 mimotope was expressed on each of the 180 coat protein subunits of the CMV, 2R9-CMV was produced expressing a second copy of the R9 sequence in another portion of the exposed region of the CMV CP. 2R9-CMV chimera was found to successfully replicate in the infected host. Besides, it was recognized robustly by sera from HCV patients. The 2R9-CMV also augmented interferon-g generation by PBMCs from HCV patients in vitro. Both R9-CMV and 2R9-CMV were highly stable to gastric and intestinal environments. Therefore, the CMV-based chimeras could be produced in a bioreactor system in order to obtain stable oral vaccines [113,119,120]. The mimotope was also expressed using alfalfa mosaic virus CP P3/RNA3, tobacco mosaic virus (TMV) CP and tobacco mild green mosaic virus (TMGMV) CP as expression vectors into tobacco plants [114].These recombinant mimotopes have been proven to be therapeutic in addition to serving as a diagnostic tool.

In continuous attempts using plant viral systems as candidates for vaccine development, tobacco mosaic virus (TMV) was used for production of a chimeric protein containing the HVR1 fused to the clolera toxin B subunit (CTB). The selected amino acid of HVR1 used in the study comprised cross-neutralizing activity [121]. The produced plant-based antigens (HVR1/CTB) resulted in immunogenic reactions against HVR1 specific monoclonal antibodies as well as the sera obtained from individuals infected with four different HCV genotypes. Furthermore, the mice which were immunized with the extract of transformed plants expressing HVR1/CTB protein triggered production of antibodies against CTB and HVR1 [121].

Aside from plant's viral systems, the whole system of plants has been studied for development of vaccine against HCV. This strategy allows plants to express recombinant proteins triggering immune response in mammalians. The Hepatitis B virus surface antigen (HBsAg)- based plasmo-virus like particles was shown as an encouraging platform for production of poly-CTL epitopes vaccines against HCV [122]. In a study, using a potato virus X-based vector (PVX), the HBsAg was fused with a polytope construct of HCV (HCVpc). The transient expression of the poly-topic construct was confirmed in tobacco plant [123]. However, in this specific study, the immunogenic response of the derived protein was not tested to evaluate the further implications. Tobacco is an excellent system for such studies because of its high susceptibility as a host for production of recombinant proteins, the high yield mass of leaves and a well known studied model system in biology. In another study, the HVR1 epitope

of HCV (R9) was cloned into the coat protein (CP) of Alfalfa Mosaic Virus (ALMV), making a chimeric region of ALMV RNA4 along with R9 epitope coding sequence. The construct then was introduced into the ALMV RNA3 vector and transformed into the tobacco plants expressing ALMV RNA1 and 2. The produced HVR1/ALMV-CP was reactive to ALMV-CP and HVR1 specific monoclonal antibodies and the sera from HCV infected patients [124].

In the last twenty years there have been different studies focusing on seed-based platforms for production of immunogenic proteins in various plants such as legumes, cereals, oilseeds and canola [125]. Seeds are also capable of high recombinant protein accumulation as they contain a higher protein content (20%) compared to the green tissues (which only contain1%-2% protein) [126]. In addition, oil-bodies may offer a great natural cargo system for storage and delivery of recombinant proteins [127]. A truncated core protein of HCV was expressed in canola for further analysis of a new vaccine platform. The study showed that a 15 kDa truncated HCV core protein was expressed in transgenic canola plants. The content of antigenic protein was estimated as 0.05% of the total soluble protein [125]. It is worth mentioning that production of seeds from transgenic T0 lines have been faced with difficulties in different studies [126,128]. Therefore, techniques should be developed to overcome those problems, resulting in a high yield production of antigenic proteins in plant seeds.

7.1. The structure of Hepatitis A Virus (HAV)

Hepatitis A virus belongs to the *Picornaviridae* family, in the genus *Hepatovirus*. The 7.5 kb genome of Hepatitis A virus (HAV) is a single plus-strand RNA with no lipid envelope. The capsid proteins are icosahedral in shape and the genome shows a high level of conservation among different genotypes. Translation is cap-independent and controlled with an internal ribosome binding site. Translation results in a single polyprotein which is finally cleaved by a viral peptidase called 3C. The peptidase 3C produces four capsid proteins (VP1- VP4) and six non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D). VP1 capsid protein comprises the main antigenic epitope of HAV. Neutralizing activity of the virus is associated with an antigenic domain where different antigenic epitopes are merged [129,130]. The antigenicity and neutralization activity of HAV necessitate production of viral particles or virions which are structured by capsid assembly [131].

7.2. Characteristics of HAV Infection

Each year around 1.5 million new HAV infections are reported across the world [132]. HAV symptoms differs from moderate to serious. The infected person might experience fever, appetite loss, malaise, nausea, diarrhea, abdominal pain, jaundice and dark urine [133]. The infection is generally transmitted through the fecal-oral route and contaminated food, water or poor quality hygienic products [134]. The stability of HAV at ambient, higher temperatures and acidic environments makes transmission of the virus much easier through water or food

products [130]. To prevent the infection, maintaining higher standards of hygiene in food, water and healthcare systems are recommended. The higher rate of disease is diagnosed in developing regions compared to developed countries [132]. The incubation period for the virus is reported to be 3 to 6 weeks [134-137]. During this period the virus replicates in liver cells [133]. Because of the long-incubation period in cells, detection is restricted to sensitive molecular techniques [130]. The severity of HAV varies depending on age of infected individuals. For instance, when infection occurs, over 80% of children under the age of five are asymptomatic, while more than 70% of infected adults are symptomatic [132,138].

7.3. Efforts towards development of plant-based vaccine against HAV

Research to gererate an effective plant-based HAV vaccine has been slow to develop for other reasons, including the lack of medical urgency due to the low number of fatalities resulting from this virus, as well as the presence of an efficacious conventional vaccine. There have been some attempts towards using plants as an alternative source of HAV vaccine. Production of HAV-VP1 recombinant protein using tomato and tobacco plants are examples where the mice immune system was found to be responsive in generating anti-HAV antibodies [129, 139]. Nonetheless, work to utilize plants as production platforms will continue to progress, with the prospect that alternative treatments low in cost and lacking refrigeration requirements would be a definite asset for developing countries, where conventional vaccines can be poorly accessible due to geographic and financial constraints.

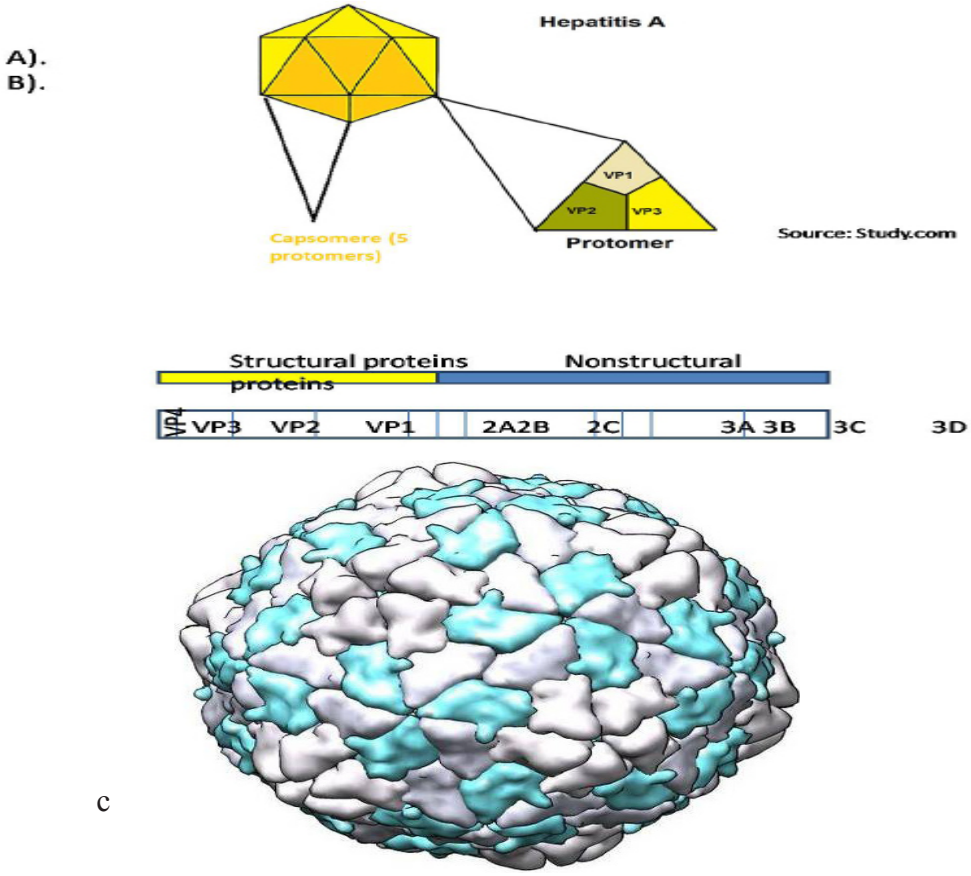

Figure 6: A). Hepatitis A Virus structure. B). Genomic organization of Hepatitis A Virus. C). Crystal structure of empty Hepatitis A Virus particle (source: Protein Data Bank).

8. References

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