Vaccine xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Oral administration of a chimeric Hepatitis B Virus S/preS1 antigen produced in lettuce triggers infection neutralizing antibodies in mice

Mihaela-Olivia Dobrica [a], Catalin Lazar [a], Lisa Paruch [b], André van Eerde [b], Jihong Liu Clarke [b], Catalin Tucureanu [c], Iuliana Caras [c], Sonya Ciulean [c], Adrian Onu [c], Vlad Tofan [c], Alexandru Branzan [d], Stephan Urban [e], Crina Stavaru [c,*], Norica Branza-Nichita [a,*]

[a] Institute of Biochemistry of the Romanian Academy, Bucharest, Romania
[b] NIBIO – Norwegian Institute for Bioeconomy Research, Ås, Norway
[c] "Cantacuzino" National Research Institute, Bucharest, Romania
[d] Institute of Biology of the Romanian Academy, Bucharest, Romania
[e] University Hospital Heidelberg, Germany

ARTICLE INFO

Article history:
Received 26 February 2018
Received in revised form 16 July 2018
Accepted 29 July 2018
Available online xxxx

Keywords:
Hepatitis B Virus
Edible vaccine
Plant expression
Neutralizing antibody

ABSTRACT

Hepatitis B Virus (HBV) infection can be prevented by vaccination. Vaccines containing the small (S) envelope protein are currently used in universal vaccination programs and achieve protective immune response in more than 90% of recipients. However, new vaccination strategies are necessary for successful immunization of the remaining non- or low-responders. We have previously characterized a novel HBV chimeric antigen, which combines neutralization epitopes of the S and the preS1 domain of the large (L) envelope protein (genotype D). The S/preS1$^{21-47}$ chimera produced in mammalian cells and *Nicotiana benthamiana* plants, induced a significantly stronger immune response in parenterally vaccinated mice than the S protein. Here we describe the transient expression of the S/preS1$^{21-47}$ antigen in an edible plant, *Lactuca sativa*, for potential development of an oral HBV vaccine. Our study shows that oral administration of adjuvant-free *Lactuca sativa* expressing the S/preS1$^{21-47}$ antigen, three times, at 1 μg/dose, was sufficient to trigger a humoral immune response in mice. Importantly, the elicited antibodies were able to neutralize HBV infection in an NTCP-expressing infection system (HepG2-NTCP cell line) more efficiently than those induced by mice fed on *Lactuca sativa* expressing the S protein. These results support the S/preS1$^{21-47}$ antigen as a promising candidate for future development as an edible HBV vaccine.

1. Introduction

Chronic infection with Hepatitis B Virus (HBV) is the primary cause of liver cancer worldwide and is responsible for the death of more than 750,000 people each year [1]. In the majority of cases, current antiviral therapies, including immunomodulators (e.g Peg-IFNa) and administration of replication inhibitors of reverse transcription (e.g. Tenofovir) are not able to cure the disease. Immunization is the most efficient measure to control HBV infection. Commercial vaccines are mainly based on the expression of the HBV small (S) envelope protein in yeast, leading to formation of highly immunogenic subviral particles (SVPs) [2]. These vaccines induce a protective immune response in most, but not all vaccinated people, with about 10% remaining at risk of infection [3]. In addition, the immunity induced by HBV vaccination may be lost in 10% of cases, 15 years after immunization [4]. Alternative HBV antigens or improved adjuvants are needed to enhance this response in the low- or non-responders and prolong persistence of the vaccine-induced immunity. Inclusion of the other two envelope proteins, middle (M) and large (L) in the standard HBV vaccine is one of the strategies adopted to overcome these issues. However, despite demonstrated enhanced immunogenicity, the high-production costs of these proteins in mammalian cells, as well as marketing policies preclude their widespread use [5].

We have recently developed a novel HBV antigen that combines relevant immunogenic and structural domains of the L and S proteins (genotype D), by insertion of amino acid residues 21–47 of the preS1 domain (21-PAFRANTANPDWDFNPNKDTWPDANKV-47)

Abbreviations: Abs, antibodies; ER, endoplasmic reticulum; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

* Corresponding authors at: Institute of Biochemistry of the Romanian Academy, Splaiul Independentei 296, Sector 6, Bucharest, Romania (N. Branza-Nichita).
E-mail address: nichita@biochim.ro (N. Branza-Nichita).

https://doi.org/10.1016/j.vaccine.2018.07.072
0264-410X/

[6] within the antigenic loop (AGL) of the S protein, between residues 126 and 127. The resulting S/preS1$^{21-47}$ chimera triggered significantly increased humoral and cellular immune responses in parenterally vaccinated mice, when compared to the S protein [7]. This antigen was successfully expressed in *Nicotiana benthamiana* plants, as a more cost-efficient recombinant protein production system and shared similar immunogenic properties with that produced in mammalian cell culture [7]. In this work we investigated the possibility to efficiently produce the S/preS1$^{21-47}$ antigen in an edible plant, as a potential candidate for oral delivery. Lettuce (*Lactuca sativa*) was chosen as expression plant host, as it is very easy to cultivate and suitable for lyophilisation, ensuring long-term stability of expressed antigens [8,9]. We have previously shown that the Hepatitis C Virus E1E2 heterodimer could be produced in lettuce and was able to trigger an immune response and antibody production when orally administered in mice [10]. Moreover, wild-type HBV envelope proteins have been successfully produced in this plant before [11].

Our study shows that the S/preS1$^{21-47}$ antigen expressed in *Lactuca sativa* induced a superior immune response in orally-vaccinated mice, as compared to the S protein. Most importantly, the elicited antibodies were able to neutralize HBV infection in an NTCP-expressing infection system (HepG2-NTCP cell line) promoting this novel antigen as a promising candidate for future development of an edible HBV vaccine.

2. Materials and methods

2.1. Cell lines, viruses and plasmids

HepG2.2.15 cells (provided by Dr. David Durantel, INSERM U871, Lyon, France) were cultured as published elsewhere [12,13]. HBV was purified from HepG2.2.15 cell supernatants and quantified as described [14]. HepG2^{hNTCP} cells (received from Prof. Stephan Urban, University Hospital Heidelberg, Germany) were maintained in culture and infected with HBV as previously described [15]. Details of plasmids coding for the S and S/preS1$^{21-47}$ antigens were published before [7,13]. The HBV-S and HBV-S/preS1$^{21-47}$ encoding genes were transferred to the plant transient expression vector pEAQ-HT-DEST1 [16] using Gateway cloning technology, as published [7].

2.2. Expression of HBV antigens in Lactuca sativa

ElectroMAX^{TM} *Agrobacterium tumefaciens* LBA4404 cells (Invitrogen, USA) were transformed with the plant expression vectors pEAQ-*HT*-DEST1/HBV-S and pEAQ-*HT*-DEST1/HBV-S/preS1, by electroporation, as described [17]. Positive clones were culture-enriched and prepared to infiltrate 4–6-week old *Lactuca sativa* plants, by employing a vacuum-based protocol, as described [16]. Transformed plants were harvested at different days post-agroinfiltration (dpi).

2.3. Antigen extraction from Lactuca sativa leaves and characterization

Lactuca sativa leaves were ground into powder in liquid N_2 and extracted for 30 min on ice in a buffer containing 0.15 M NaCl, 20 mM Na_2HPO_4, 20 mM sodium ascorbate, 0.5% Triton X-100 (pH 7) and a protease inhibitors cocktail (Santa Cruz Biotechnology). Extracts were cleared by successive centrifugations for 5 min at 1000*g* and 15 min at 27,000*g*, at 4 °C. Supernatants were either analyzed for the presence of the HBsAg (Hepatitis B surface antigen) by ELISA, using the Monolisa HBsAg ULTRA kit (Bio-Rad), or subjected to western blotting. To determine their SVP content, plant samples were 20-fold concentrated by ultracentrifugation on 20% sucrose for 5 h, at 32,000 rpm (SW 32Ti rotor), along with HepG2.2.15 cell supernatants, as control. The PBS-dissolved pellets were layered onto a step sucrose gradient (15, 25, 35, 45 and 60% sucrose) and centrifuged for 16 h at 30,000 rpm (SW41 Ti rotor). Fractions (750 μl) were analyzed for the HBsAg content by ELISA, using the same kit as above [6].

2.4. Transmission electron microscopy (TEM) analysis

HBsAg-positive fractions collected from the sucrose gradient were dialyzed against PBS and further concentrated by lyophilisation. Samples were deposited on formvar film-coated copper grids and analyzed by TEM, using the JEM-1400 microscope (Jeol).

2.5. SDS-PAGE and western-blotting

SDS-PAGE and western blotting were performed as published previously [13]. Proteins were detected with mouse anti-preS1 antibodies (Abs) (sc-57762 mc, Santa Cruz, 1/1000), followed by rabbit anti-mouse HRP-conjugated Abs (Santa Cruz, 1/10,000).

2.6. Animals and immunization schedules

Experiments were performed using 6–8-week-old female Balb/c mice. All animal care was in accordance with standards set forth in the Council Directive 86/609/EEC, and protocols were approved by the Internal Ethics Committee of the "Cantacuzino" Institute. Mice were divided in 4 groups, each containing 5 animals. The first group was immunized twice with 1 μg of Engerix (Glaxo Smith Kline Biological SA) injected into the quadriceps muscles (im), at a two-week interval. Two other groups received lettuce expressing either the S/preS1$^{21-47}$ or the S antigen at ~1 μg/dose, orally, three times at a one-week interval. The oral immunization with each antigen consisted in individual feeding of mice, overnight (*ad libitum*), with a pellet weighing between 4 and 6 g, obtained by mixing the appropriate amount of lettuce with standard chow powder. Mice were individually monitored for consumption of lettuce pellets. A fourth animal group including non-immunized mice was also included as control. On day 69, the orally immunized mice groups received an extra feeding boost with lettuce powder containing either the S/preS1$^{21-47}$ or S antigen at ~1 μg/dose. Blood samples and fecal pellets were collected on days 14, 28, 42, 56 and 70 post-immunization, and analyzed as previously indicated [7].

2.7. Quantification of the cellular immune response

Mice were anaesthetized (by intraperitoneal administration of a mixture containing Ketamine 100 mg/kg and Xylazine 10 mg/kg) and euthanized by cervical dislocation. Spleens were harvested and splenocytes were prepared, cultured and stimulated with either HBV or PBS, as described [7]. Antigen-specific IL-5- and IFN-γ-secreting cells were quantified by ELISPOT, using the Mouse IFN-γ/IL-5 FluoroSpot kit (Mabtech) as shown previously [7].

2.8. Quantification of the humoral immune response

Serum and feces samples were 2-fold serially diluted in PBS containing 5% nonfat dry milk then added to flat bottom 384-well MediSorp plates (Sigma-Aldrich), coated with 0.162 μg/ml (20 μl/well) of an inactivated HBV suspension in PBS. Sample analysis was carried out by ELISA, as described [7]. Normalization across ELISA plates was performed by pooling all the immune sera from day 56 which served as internal standard. Serum pool dilution starting from 1/100 was used as internal standard on each plate and a 4-parameter logistic regression model was fitted to

Please cite this article in press as: Dobrica M-O et al. Oral administration of a chimeric Hepatitis B Virus S/preS1 antigen produced in lettuce triggers infection neutralizing antibodies in mice. Vaccine (2018), https://doi.org/10.1016/j.vaccine.2018.07.072