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Development of a plant-derived subunit vaccine candidate against hepatitis C virus

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Summary. Hepatitis C virus (HCV) is a major cause of acute and chronic hepatitis with over 180 million cases worldwide. Vaccine development for HCV has been difficult. Presently, the virus cannot be grown in tissue culture and there is no vaccine or effective therapy against this virus. In this research, we describe the development of an experimental plant-derived subunit vaccine against HCV. A tobamoviral vector was engineered to encode a consensus sequence of hypervariable region 1 (HVR1), a potential neutralizing epitope of HCV, genetically fused to the C-terminal of the B subunit of cholera toxin (CTB). This epitope was selected from among the amino acid sequences of HVR1 "mimotopes" previously derived by phage display technology. The nucleotide sequence encoding this epitope was designed utilizing optimal plant codons. This mimotope is capable of inducing cross-neutralizing antibodies against different variants of the virus. Plants infected with recombinant tobacco mosaic virus (TMV) engineered to express the HVR1/CTB chimeric protein, contained intact TMV particles and produced the HVR1 consensus peptide fused to the functionally active, pentameric B subunit of cholera toxin. Plant-derived HVR1/CTB reacted with HVR1-specific monoclonal antibodies and immune sera from individuals infected with virus from four of the major genotypes of HCV. Intranasal immunization of mice with a crude plant extract containing the recombinant HVR1/CTB protein elicited both anti-CTB serum antibody and anti-HVR1 serum antibody which specifically bound to HCV virus-like particles. Using plant-virus transient expression to produce this unique chimeric antigen will facilitate the development and production of an

experimental HCV vaccine. A plant-derived recombinant HCV vaccine can potentially reduce expenses normally associated with production and delivery of conventional vaccines.

Introduction

The use of plants to produce vaccine antigens for human diseases is a novel and promising system with several practical advantages compared to fermentation or cell-culture facilities such as delivery of edible vaccines by the oral route, the ease of agricultural scale production, safety from contaminating human or animal pathogens, and the possibility of growing and manufacturing vaccine materials locally for specific demands. Plant engineering technology is now at an exciting stage of development. Technical difficulties such as low levels of expression, antigen purification strategies, and inadequate immunogenicity of plant-derived recombinant proteins are being addressed by researchers and many innovative solutions are rapidly emerging [4, 12]. This report describes our efforts to develop a subunit vaccine candidate for hepatitis C virus (HCV) using a modified tobacco mosaic virus (TMV) to produce recombinant vaccine antigens in infected plants [26].

HCV is an important pathogen affecting more than 180 million people worldwide including nearly 4 million in the United States [3, 6, 20]. Currently, there is no vaccine or effective therapeutic agent available. A prevailing view of HCV immunity is that both humoral and cell-mediated host immune responses develop during the initial infection, but rapidly become ineffective against the highly mutating heterologous virus population that evolves during the course of the disease. The number of sites in the viral genome that have been identified as either responsible for inducing a protective immune response or mediating viral clearance is growing [8, 15, 19, 22]. It seems possible that many of the "evolving" escape epitopes are scattered throughout the HCV genome preventing the development of an effective anti-viral response [8]. Nevertheless, the importance and hierarchy of individual epitopes may be relevant to the mechanism of viral resistance to host immune response. Hypervariable region 1 of the HCV envelope protein 2 has been identified as a principal neutralization epitope responsible for viral heterogeneity [10, 19, 24, 25]. For our research, we chose a vaccine development approach that is predicted to be effective against extremely high antigen variability. Our subunit vaccine originates from a consensus HCV HVR1 epitope that antigenically mimics many natural HVR1 variants [19]. This HVR1 sequence was genetically fused to one of the most potent natural adjuvants, the B subunit of cholera toxin (CTB). This recombinant chimeric protein is expressed in plants and can be easily prepared for immunization through the mucosal route.

Material and methods

Cloning

Recombinant immunogen was obtained using a fusion PCR reaction with a primer for the 5′-terminus of the CTB gene, linker primer containing both the 3′-terminal fragment of the

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  1 ATGATTAAAT TAAAATTTGG TGTTTTTTTT ACAGTTTTAC TATCTTCAGC
 51 ATATGCACAT GGAACACCTC AAAATATTAC TGATTTGTGT GCAGAATACC
101 ACAACACACA AATACATACG CTAAATGATA AGATATTTTC GTATACAGAA
151 TCTCTAGCTG GAAAAAGAGA GATGGCTATC ATTACTTTTA AGAATGGTGC
201 AACTTTTCAA GTAGAAGTAC CAGGTAGTCA ACATATAGAT TCACAAAAAA
251 AAGCGATTGA AAGGATGAAG GATACCCTGA GGATTGCATA TCTTACTGAA
301 GCTAAAGTCG AAAAGTTATG TGTATGGAAT AATAAAACGC CTCATGCGAT
351 TGCCGCAATT AGTATGGCAA ATCAAACTAC TGTTGTTGGA GGATCTCAAT
401 CTCATACTGT TAGAGGACTT ACTTCTCTTT TTTCTCCAGG AGCTTCTCAA
451 AATAAAGATG AACTTTAA
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A

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  1  MIKLKFGVFF TVLLSSAYAH GTPQNITDLC AEYHNTQIHT LNDKIFSYTE
 51  SLAGKREMAI ITFKNGATFQ VEVPGSQHID SQKKAIERMK DTLRIAYLTE
101  AKVEKLCVWN NKTPHAIAAI SMANQTTVVG GSQSHTVRGL TSLFSPGASQ
151  NKDEL*
```

B

Fig. 1. A Nucleotide sequence of CTB/HVR1 chimeric gene. **B** Translated amino acid sequence of the recombinant protein. The HVR1 fragment is underlined

CTB gene and the 5′-terminal portion of the R9 HVR1 mimotope coding sequence [19], and the complementary primer for the 3′-portion of the HVR1. Products were cloned into a pCR-Blunt II Topo vector (Invitrogen) and then consecutively incorporated into the *Xho* I site of TMV-based hybrid vector 30B [26]. Dr. Shailaja Shivprasad from Dr. William O. Dawson's laboratory at the University of Florida, Lake Alfred, FL kindly supplied the 30B vector. The gene for the CTB subunit was derived from a subclone of plasmid pRT41 [17] kindly provided by Dr. John J. Mekalanos, Harvard University, Boston, MA. Extreme difficulties encountered during of routine cloning into the 30B vector were overcome by using MAX Efficiency Stbl2 competent cells from Gibco BRL, which gave 80–90% cloning efficiency. The recombinant gene was sequenced to insure that the correct fusion protein was encoded (ABI-PRIZM 373A Genetic Analyzer; DNA sequencing facility at the University of Maryland, Center for Agricultural Biotechnology at College Park, MD). The sequence of the R9 HVR1 mimotope used is shown in Fig. 1.

In vitro transcription and plant inoculation

Capped transcripts from full-length recombinant TMV cDNA clones were generated with the T7 Cap Scribe kit (Boehringer Mannheim). Reaction mixture was diluted 1:1 in 20 mM sodium phosphate buffer pH 7.2 and used to inoculate the three top leaves of *Nicotiana benthamiana* plants that were at the four-leaf stage.

RT-PCR and protein analysis

Total RNA was extracted from systemically infected, uninoculated leaves with symptoms by RNAeasy Plant Mini Kit (Qiagen) and used for conventional reverse transcription-polymerase chain reaction assays (RT-PCR) and Northern blot analysis. For Western blots, 50–100 mg of plant leaf samples were homogenized in 1×PBS containing 1% plant protease inhibitor cocktail (Sigma), centrifuged at 14 krpm for 10 min at 4 °C in an Eppendorf centrifuge. The supernatant fraction was mixed 1:1 with Novex native sample buffer (Novex) and proteins were analyzed by 10–20% Tris-glycine PAGE (Novex) and immunoblots using chemiluminescent detection. In some instances, samples were concentrated using Centriprep YM-10 concentrators (Millipore) according to manufacturer's instructions. Total protein concentration of the plant samples was determined by Coomassie dye binding assay (Bio-Rad) with bovine serum albumin as a standard.

For CTB, monoclonal antibodies were purchased from Research Diagnostic Inc. (cat. # RDI-TRK2C4) and used at 1:2,000 dilution; polyclonal antibodies were from Calbiochem (cat # 227040) and used at 1:10,000 dilution. Dr. Jane McKeating of University of Reading and Dr. Arvin Patel of MRC, Glasgow, UK kindly provided three different monoclonal antibodies for HVR1. Both the 6/82a and 6/82b MAbs recognize epitope ETHVTGGS, aa 384–391 and MAb ALP213, recognizes epitope GAARSTLQLAGLFQPGAKQN, aa 390–409. Sera of HCV genotypes 1a, 1b, 2a, 2b, 3a, and 3b were from patients followed at the Liver Disease Section, NIDDK, NIH. Genotype 4a is the predominate HCV strain in Egypt. The serum that we used for this genotype was from an HCV infected Egyptian patient obtained through Express Blood Test Laboratory at Johns Hopkins University, Baltimore, MD. MAbs were used at 1:5,000 dilution and sera at 1:20,000 dilution.

ELISA assay

For ELISA, plates were coated at 100 μl per well for 2 h at 37 °C with a 1:10,000 dilution of anti-CTB polyclonal goat antiserum in carbonate buffer (50 mM N_2CO_3, pH 9), then blocked with 1% BSA solution for 1 h at 37 °C. Plates then were incubated overnight with the plant samples at 4 °C, followed by incubation with anti-CTB MAbs at 37 °C for 2 h. All washing steps between incubations were performed with 1×PBS-Tween buffer. After room temperature incubation for 1 h with anti-mouse IgG-HRP conjugate, horseradish peroxidase substrate was added to the wells. Plates were read at 405 nm and the data analyzed by SoftMax. For chemiluminescent ELISA, SuperSignal ELISA Femto Maximum Sensitivity substrate was used (Pierce). The plates were read and relative light unit values measured in a ML3000 plate Luminometer (Dynex).

GM1-ELISA assay

For GM1-ganglioside ELISA, plates were coated at 37 °C for 2 h with GM1 monoganglioside (Sigma) at 1 μg/ml diluted in carbonate buffer. The remaining procedure was similar to the ELISA test described above. The GM-1-monoganglioside receptor only recognizes the CTB pentamer configuration; thus the bound CTB pentamers were then identified by using either monoclonal or polyclonal anti-CTB antiserum and HRP-secondary antibody conjugate. To estimate the levels of recombinant pentameric CTB protein produced in plant tissue, the O.D. values of the enzyme-substrate reaction at 405 nm from known amounts of authentic cholera toxin B subunit were compared to the plant-derived CTB-protein.

Electron microscopy

TMV particles from crude leaf extracts of infected tissue were trapped on carbon-coated grids coated with anti-TMV antibodies at 1:1000 dilution according to Derrick [5]. Bound

virus particles were then coated with TMV antiserum at 1:100 dilution, stained with 2% uranyl acetate and examined using a JEOL 100-CXII electron microscope at a magnification of 40,000-80,000.

Immunization of mice

All animal protocol followed NIH guidelines. Pathogen-free C57B1/6 female mice eight weeks of age at time of immunization were used. Recombinant immunogen was administered intranasally in 30 μl dose, 15 μl for each nostril on days 0, 7, 14, 21, and a final immunization on day 69. Immunogen was a YM-10-concentrated plant extract in 1×PBS. One μg of authentic CTB (Sigma) was administered as a positive control. Mice were bled from the tail vein on day 28 and day 74.

Results

Construct engineering and nucleotide sequencing

The recombinant immunogen was assembled from the coding region of cholera toxin B subunit and the HVR1 sequence reported elsewhere [19]. For our experiments, we chose cross-reactive HVR1 mimotope R9 which antigenically mimics a large number of natural HCV HVR1 variants and induces a broadly cross-reactive anti-HVR1 response [19]. The sequence encoding the 27 amino acid-long HVR1 mimotope (designed using optimal plant codons) was linked to the carboxy-terminus of the CTB gene by fusion PCR. DNA sequencing confirmed the incorporation of the fusion gene into the B30 TMV vector (Fig. 1). C-terminal positioning of fusion peptides has been shown to provide the most

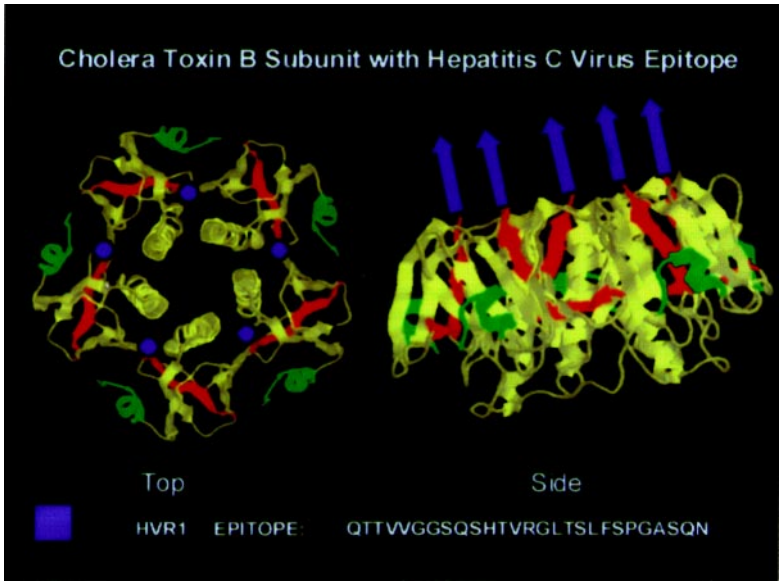


Fig. 2. Ribbon structure of the CTB pentamer carrying HVR1 epitope of HCV envelope protein 2. Vertical arrows indicate the epitope fusion. The pentamer model is from the Brookhaven Protein Data base (code 2CHB) and was drawn using the RasWin Molecular Graphics version 2.6 program

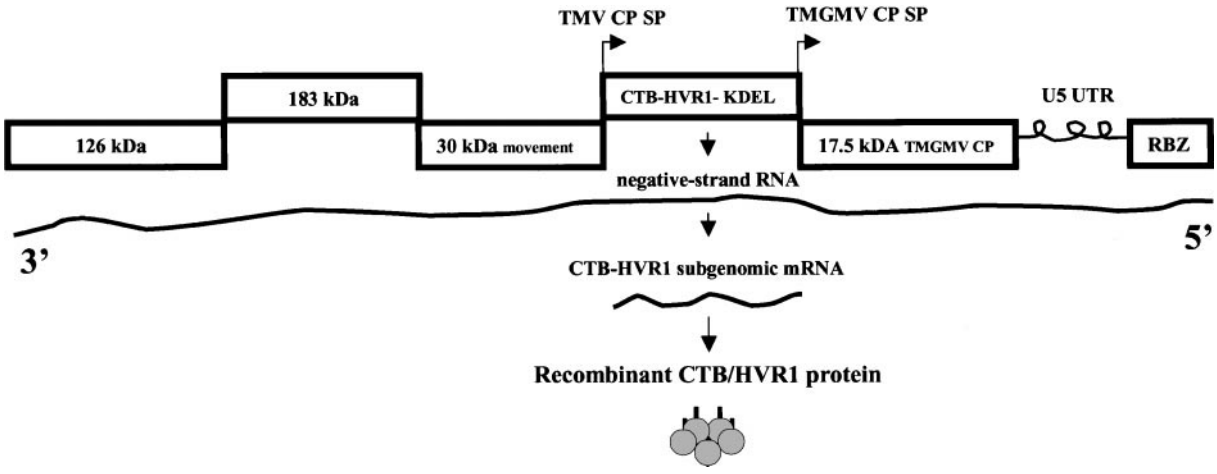


Fig. 3. Cartoon drawing showing a schematic diagram of the hybrid TMV-based vector carrying recombinant CTB-HVR1 gene. 126 kDa, 183 kDa: proteins required for TMV replication; 30 kDA: movement protein; TMV CP SP: subgenomic promoter of TMV coat protein; TMGMV CP SP and 17.5 kDa TMGMV CP: subgenomic promoter and heterologous coat protein of tobacco mild green mosaic virus variant U5; U5 UTR: 3′ untranslated region of TMGMV; RBZ: rybozyme

efficient CTB pentamerization and exposure of foreign epitopes [16, 21]. The KDEL motif was added to the HVR1 C-terminus in order to increase the yield of recombinant protein in the plant tissue by retaining the protein in the endoplasmic reticulum (ER) and protecting it from proteolytic activity [23]. The recombinant protein pentameric structure is presented in Fig. 2. In this model, the ribbon structure represents pentameric CTB and was derived from its crystal structure. A cartoon drawing representing the recombinant TMV vector, the genomic and subgenomic RNAs, and the CTB/HCVR1 fusion protein pentameric structure is presented in Fig. 3. The recombinant immunogen carrying a CTB leader sequence at the N-terminus and a KDEL retention signal at the C-terminus was placed under control of the TMV coat protein (CP) promoter. The assumption was that CTB/HVR1 subgenomic message transcribed from recombinant viral RNA will be translated into individual polypeptides and then assemble into pentameric CTB particles displaying the HVR1 epitope on their surface (Figs. 2 and 3).

Infectivity of recombinant TMV

In-vitro synthesized capped RNA transcripts of the recombinant TMV were used to inoculate the three upper leaves of *Nicotiana benthamiana* plants that were at the four-leaf stage. Symptoms indicative of TMV infection were first visible as mild yellow spots at 5–7 dpi. Plants then became systemically infected developing typical green mosaic and severe curling of the leaves at 14–21 dpi. Samples from plants infected with the recombinant TMV vector were examined using immuno-

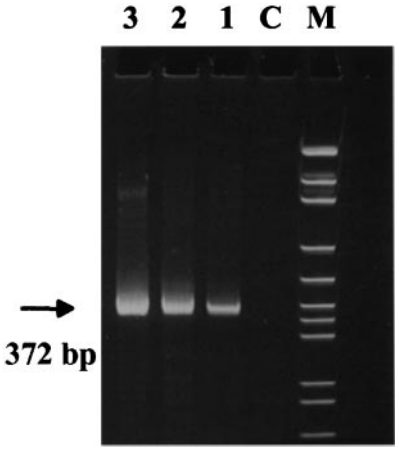


Fig. 4. RT-PCR with total RNA from *N. benthamiana* plants inoculated with hybrid TMV vector. Primers specific for B subunit of cholera toxin were used. *M* D15 Novex DNA marker, 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, and 126 bp; *C* RNA from uninfected plant; *1,2,3* Different plants infected with recombinant TMV

electron microscopy. TMV particles of regular morphology were observed in treated plants indicating that the in vitro synthesized chimeric viral RNA transcript was infectious (data not show).

Reverse transcription-PCR (RT-PCR) and Northern blot hybridization

To confirm that the TMV RNA from infected plants indeed carries the engineered construct, we performed a RT-PCR assay utilizing CTB-specific primers and total RNA isolated from infected *N. benthamiana* plants. An amplified fragment of the expected size (372 bp) was obtained from several *N. benthamiana* plants infected with the recombinant virus (Fig. 4). In addition, digoxygenin-labeled cRNA probes were prepared using T7 RNA polymerase and DNA template from either the CTB gene or the HVR1 coding sequence. In Northern blot analysis, these probes hybridized with RNA samples extracted from infected plants but not from identical uninfected control plants (data not shown). These experiments, together with the immuno-electron microscopy experiments, demonstrate that TMV RNA is infectious, replicates in the tobacco host, and contains the engineered sequence encoding the CTB/HVR1 antigen.

ELISA tests

ELISA tests using anti-CTB polyclonal and monoclonal antibodies clearly demonstrate that *N. benthamiana* plants infected with recombinant TMV transcripts produce the B subunit of cholera toxin (Fig. 5A). To prove that the CTB subunit carries the HVR1 fusion on its surface, we performed an ELISA assay using anti-CTB polyclonal antiserum as primary antibodies to capture the CTB/HVR1 protein on the ELISA plates and a mixture of three anti-HVR1 monoclonal antibodies to specifically detect the HVR1 epitope on the captured chimeric protein. As shown in Fig. 5C, only plants infected with the recombinant TMV were positive indicating that the HVR1 epitope is indeed associated with the CTB subunit (Fig. 5C).

GM1-ELISA

Biological functions of CTB, such as binding capacity to its GM1-ganglioside cell receptor, depend on the formation of a pentamer configuration by the CTB subunits. To test if the CTB subunits produced in plants form pentamers, a GM-1 ELISA assay was used. ELISA plates were coated with GM-1 receptor and authentic CTB (Sigma) was used as a positive control. The GM1-ELISA clearly demonstrated that plant-produced chimeric CTB/HVR1 protein is present in the plant cells in the active pentameric form (Fig. 5B).

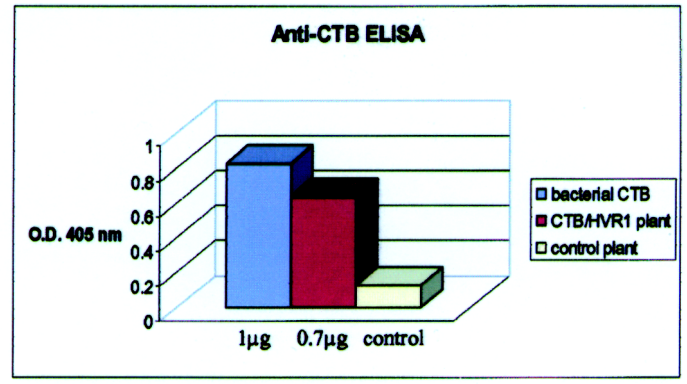
Western blot analysis of the recombinant CTB-HVR1 gene product

It has been previously shown that non-covalent interactions between CTB subunits of the pentamer are strong enough to allow analysis of the intact pentamer molecule by SDS-PAGE under non-reducing conditions [16]. Plant samples were analyzed for the formation of CTB-pentamers using Novex Tris-glycine 10–20% gels, Novex native sample buffer (which contains neither SDS nor reducing agent), and Tris-glycine-SDS running buffer.

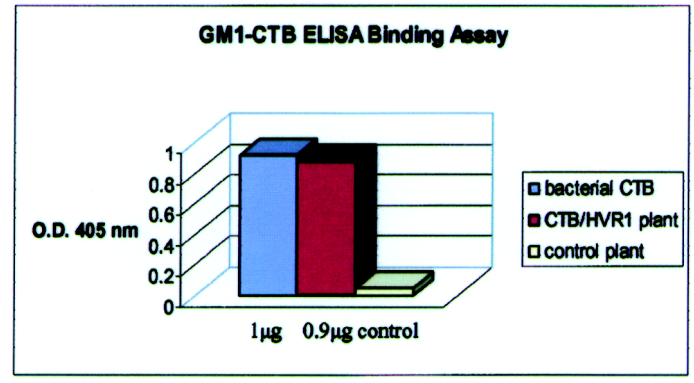
Immunoblot analysis utilizing CTB-specific MAbs revealed a predominate protein band corresponding to the putative 50 kDa CTB/HVR1 (Fig. 6a, panel B). Identical results were obtained when anti-CTB polyclonal antibodies were used (data not shown). An HVR1 peptide would increase the 45 kDa of native CTB pentamer to approximately 50 kDa, which corresponds to the observed size of chimeric protein. Thus, it appears from the increase of molecular mass that the CTB pentameric ring contains the HVR1 fusion. To finally confirm this observation, immunoblots were probed with sera from individuals infected by different genotypes of HCV as well as with several monoclonal antibodies against HVR1. In these experiments, sera from patients infected with HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, or 4a reacted with plant extracts infected with recombinant CTB/HVR1 TMV, producing a band with molecular size identical to the band visualized using CTB antibodies (50 kDa) (Fig. 6b). In another experiment, an immunoblot with YM-10-concentrated infected plant samples reacted strongly with the mixture of three HVR1 MAbs (Fig. 6c, panel A). In addition, each of the individual HVR1 MAbs also bound specifically to the CTB-HVR1 protein (Fig. 6c, panel B–D). These data indicate that an HVR1 mimotope is expressed as a fusion to the CTB.

Quantification of chimeric protein concentration

Using colorimetric GM1-ELISA assays, the amount of recombinant pentameric CTB protein in an infected plant extract was measured by comparison of the O.D. values at 405 nm with known concentrations of authentic bacterial CTB. In these experiments, approximately 100 μg of total soluble protein (TSP) obtained from 12.5 mg of leaf tissue of an infected plant was loaded per ELISA plate well. Based on this method and also based on the results of CTB-ELISA experiments, the amounts of chimeric CTB per 100 μg of total soluble protein varied within a range of 70–950 ng, which is 0.07–0.9% of TSP and approximately



A



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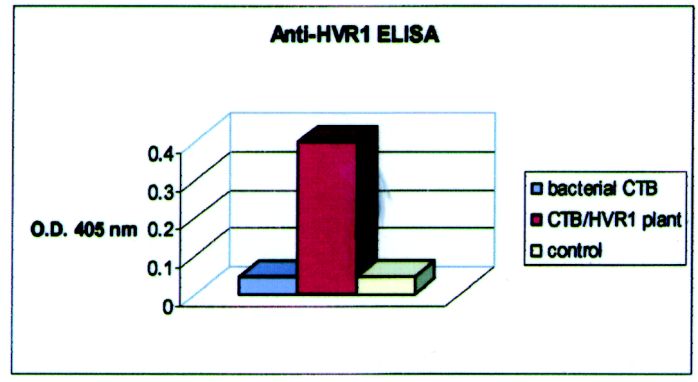

C

Fig. 5. CTB-specific ELISA with recombinant TMV/CTB/HVR1-infected plants. Anti-CTB polyclonal goat antiserum was used as a primary antibody; anti-CTB monoclonal mouse antiserum was used as a secondary antibody. Cutoff OD value of 0.159 was the background signal from the buffer controls. **B** Anti-CTB GM1-ELISA assay with GM1 ganglioside as a primary capturing agent and anti-CTB MAbs as a secondary antibody. Cutoff O.D. value of 0.048 was the background signal from buffer controls. **C** HVR1-specific ELISA assay. GM1 ganglioside was used to capture the recombinant protein and mixture of anti-HVR1 MAbs was used as a secondary antibody. Cutoff O.D value of 0.046 was the background signal from buffer controls. Approximate concentration values of CTB/HVR1 in **A** and **B** (0.76 μg and 0.95 μg, respectively) were drawn from comparison with known concentrations of bacterial CTB (1 μg)

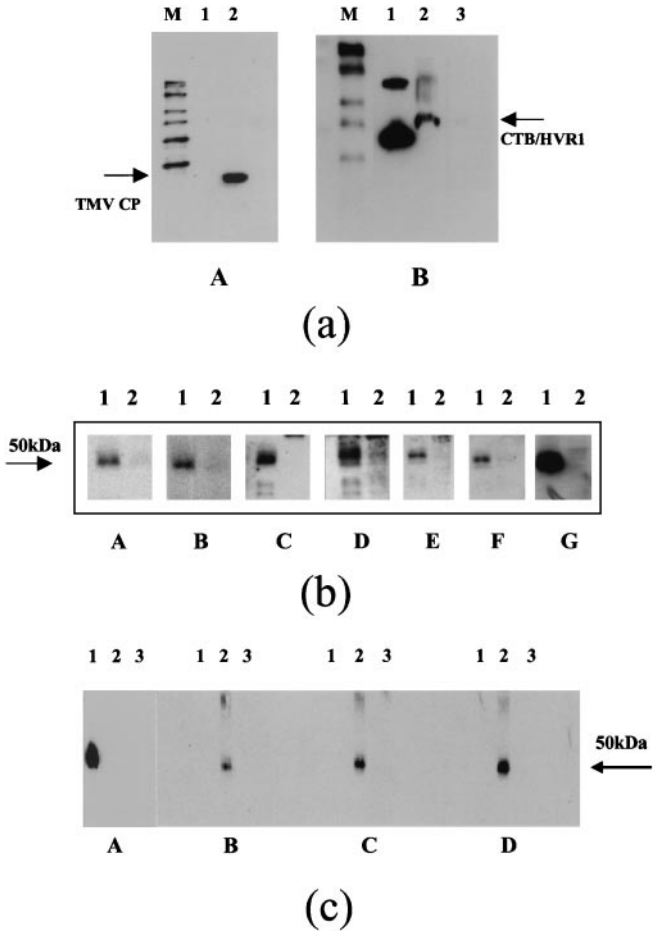

Fig. 6. a Western blot analysis of *N. Benthamiana* plants infected with recombinant TMV/CTB-HVR1 vector. *A* Membranes were probed with anti-TMV coat protein (CP) polyclonal antibodies. *M* Molecular weight standards: 132 kDa, 90 kDa, 55 kDa, 43 kDa, 34 kDa and 23 kDa (Santa Cruz Biotechnology, Inc.); *1* extract from uninfected control plant; *2* extract from plant infected with recombinant TMV. *B* Membranes were probed with anti-CTB monoclonal antibodies. *M* Molecular weight standards; *1* authentic CTB, 100 ng; *2* extract from plant infected with recombinant TMV; *3* extract from uninfected control plant. **b** Western blot analysis of CTB-HVR recombinant protein expressed in infected plant. Membranes were probed with immune sera from HCV-type 1a (*A*) HCV type 1b (*B*), HCV type 2a (*C*), HCV type 2b (*D*), HCV type 3a (*E*), HCV type 3b (*F*) and HCV type 4 (*G*). *1* CTB/HVR protein from plant infected with recombinant TMV; *2* extract from uninfected control plant. **c** Western blot analysis of CTB-HVR recombinant protein expressed in infected plant. *A* Membranes were probed with a mixture of three HVR1-specific monoclonal antibodies. *1* YM-10 filter-concentrated CTB/HVR plant extract; *2* YM-10 filter concentrated extract from control plant; *3* bacterial CTB. Membranes were also probed separately with three different monoclonal antibodies against the HVR1 region: *B* anti-HVR1 MAbs 6/82a; *C* anti-HVR1 MAbs 6/82b; *D* anti-HVR1 MAbs ALP 213. *1* CTB; *2* TMV/CTB/HVR1-infected plant; *3* control plant

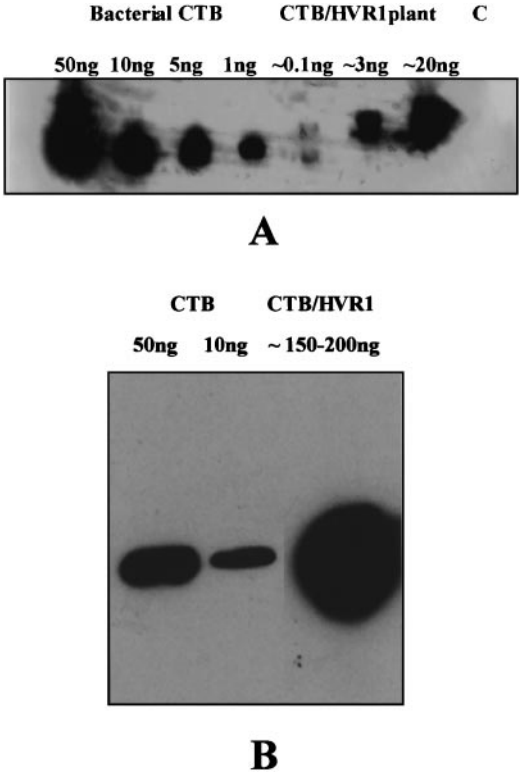

Fig. 7. Quantitative western blot analysis: estimation of protein concentration by comparison of band intensities. Anti-CTB monoclonal antibodies were used. **A** Bacterial CTB concentration ranging from 50 ng to 1 ng per load and estimated concentration of CTB/HVR1 chimera in crude extractions of leaf tissue from infected plants. **B** Dilutions of bacterial CTB, 50 ng and 10 ng; YM-10-concentrated leaf extracts from recombinant TMV-infected plant

6 to 80 μg per gram of leaf tissue. The high variability of recombinant protein content in ELISA experiments may be due to different CTB/HVR1 accumulation in randomly collected leaf samples of infected plants. The yield of recombinant CTB/HVR1 protein was additionally estimated in side-by-side comparison of authentic CTB samples of known concentration with experimental samples in Western blot assays. Direct comparison of band intensities allows simple and reliable visual estimation of protein concentration in the samples. This approach gave us an estimate amount of 200 ng of CTB/HVR1 chimeric protein per 100 μg of TSP, which is 0.2% of TSP (Fig. 7A). A 10-fold increase in protein concentration in the plant extract was obtained using Centriprep concentrators (YM-10) (Fig. 7B).

Immunization of mice and induction of serum antibodies

An immunization test was performed in order to estimate immunogenic potential of the plant-produced CTB/HVR1 recombinant protein. In this preliminary trial, a group of mice was immunized with either infected plant extract or uninfected plant extract and authentic CTB controls. Three mice were immunized intranasally with YM-10 filter-concentrated extracts from tobacco plants systemically infected with recombinant TMV. One mouse was immunized with an identically treated extract from uninfected plant and one mouse was immunized with authentic CTB (Sigma). The estimated amount of administered CTB/HVR1

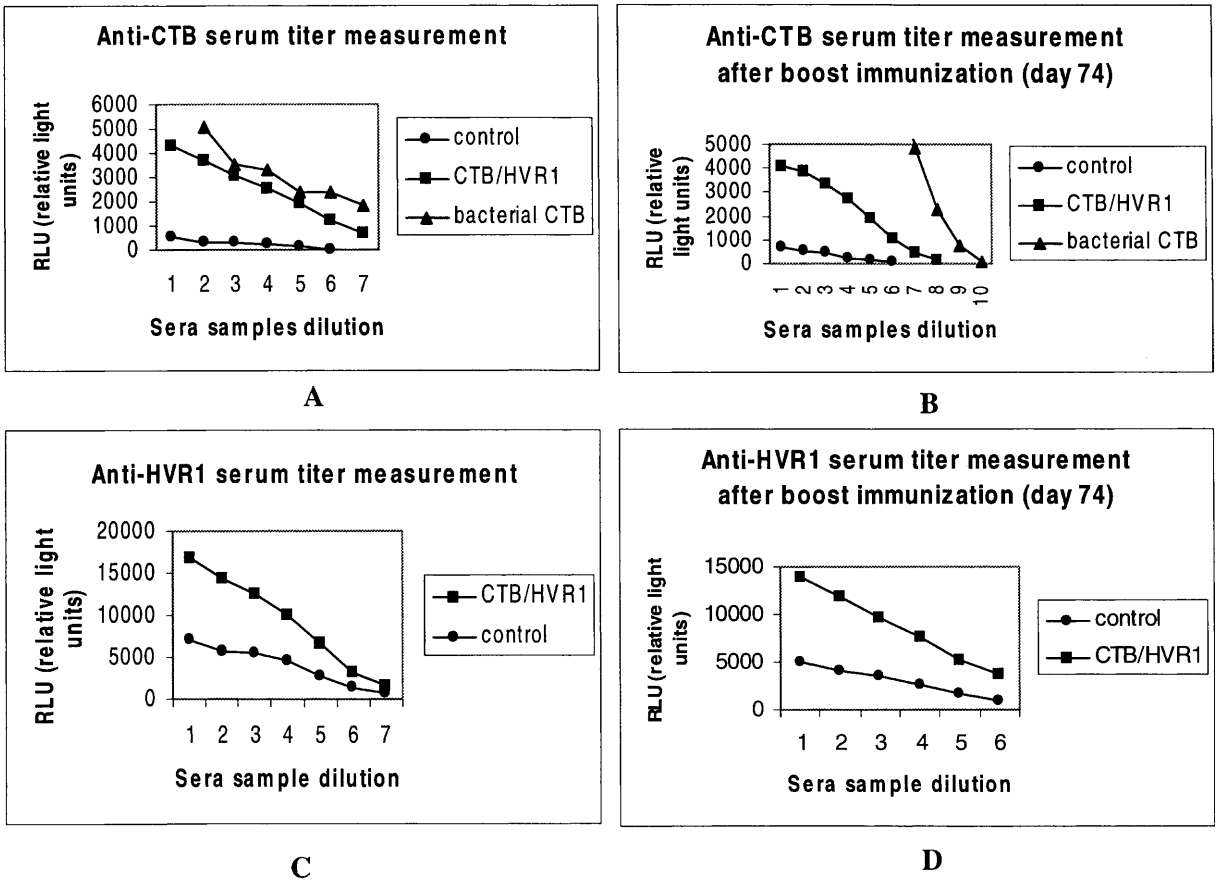

Fig. 8. Endpoint titer measurement. RLU values obtained in Dynex Microtiter Plate Luminometer. 1 through 7 or 10 are two-fold dilutions of sera from immunized mice: 1:50, 1:100. 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800 and 1: 25600 correspondingly. Control: mouse immunized with uninfected plant extracts; CTB/HVR1 averages of RLU, obtained from mice immunized with TMV/CTB/HVR1-infected plant extracts. Bacterial CTB: serum sample obtained from mouse immunized with authentic CTB. **A** Anti-CTB antibody titer measurement on day 28. Cutoff value of 524.03 is an RLU obtained from 1:50 diluted serum of control mouse. **B** Anti-CTB antibody titer measurement on day 74. Cut-off value of 700.4 is an RLU obtained from 1:50 diluted serum of control mouse. **C** HCV-LP ELISA for the detection of anti-HVR1 antibodies, day 28. Cutoff values are at RLU 7116.3, which is 1:50 dilution of control mouse serum. **D** Anti-HVR1 serum titer measurement on day 74. Cut-off values of 4912.9 RLU represent a data obtained from 1:50 dilution of control mouse serum

antigen from the plant extract was 0.5–1 μg per mouse. For serum antibody titer determination, mice were bled from the tail vein on day 28, boosted on day 69 and bled again on day 74. Sera samples were diluted two-fold in 1×PBS starting from 1:50. Chemiluminescent ELISA was performed using 5 μg/ml of authentic CTB to capture antibodies and anti-mouse IgG-HRP conjugate (Santa-Cruz) in 1:2000 dilution for the detection. Background relative light units (RLU) from wells containing only substrate were subtracted from the RLU of experimental samples.

The results of this experiment indicate that on day 28 CTB/HVR1-immunized mice developed an anti-CTB immune response comparable to the mouse immunized with authentic CTB (Fig. 8A). Serum anti-CTB antibodies reached a titer of 1:3200. The anti-CTB serum immune response in mice immunized with plant-derived CTB/HVR1 protein was high on day 74, five days after the secondary or "booster" immunization (Fig. 8B). The observed titers were on the level of 1:1600–1:3200, which suggests the establishment of immunological memory after the primary immunization.

To determine if antibodies produced in the mice to the CTB/HVR1 antigen would recognize HCV, an effective HCV virus-like particle-ELISA assay (HCV-LP) was used [2]. HCV-LPs are highly immunoreactive to anti-HCV antibodies and exist in a conformation similar to the native virion. This technique has been shown to be an effective tool for the detection of anti-HCV immune response [2]. In our experiments using the HCV-LP chemiluminiscent ELISA assay, immunized mice developed a specific anti-HVR1 immune response with an average serum antibody titer of 1:800 on both day 28 and after the second immunization on day 74 (Fig. 8C, D).

Discussion

We have explored the plant system as an inexpensive source for the production of a potential HCV vaccine candidate. Plant-synthesized recombinant CTB/HVR1 protein described in our study appears to be in the biologically active pentamer configuration (CTB) and immunoreactive for its CTB and HVR1 epitopes. Preliminary mouse immunization experiments, although done with a small cohort of mice, nevertheless demonstrate that even trace amounts of less than a microgram of non-purified recombinant protein present in infected plant extracts is capable of eliciting a high-titer anti-CTB immune response and reasonable anti-HVR1 antibody titers without any additional adjuvant. The approximate portion of the five HVR1 epitopes that make up the assembled CTB pentamer fusion protein molecule is less that 1/10; therefore, the amount of HVR1 epitope administered is less than 0.1 μg per immunization dose. These results demonstrate that this approach for vaccine development is promising. It is our opinion that larger amounts of plant-derived and specifically purified CTB/HVR1 recombinant protein will undoubtedly induce a much stronger anti-HCV immune response. In our experiments, we found that the CTB-displayed HVR1 antigenic determinant cross-reacted with different serum samples derived from HCV-infected patients representing four of the major known viral genotypes. This is a direct confirmation of immunological similarity between the R9 HVR1 "mimotope" expressed in plants and the many natural HVR1 variants. Thus, if HVR1 indeed contains a principal neutralization epitope for HCV, a plant-produced, purified HVR1 surrogate antigen will be able to induce cross-neutralizing antibodies against most HCV quasispecies.

We suggest that contribution of CTB to the antigenic properties of the linear HVR1 epitope is important and expands the HVR1 immunogenic potency. The

cholera toxin B subunit is involved in complex interactions with the immune system resulting in a significant adjuvant effect [13, 27]. Unlike other heterologous helper genes used to engineer hybrid plant virus vectors, CTB may also elicit useful anti-toxin protection in addition to its well-established adjuvant characteristics and epitope-presentation of genetically fused peptides. It is important that CTB can accommodate fusions and fully retains its native pentameric structure [21]. Binding of the CTB pentamer to its GM-1 cell receptor leads to a number of key immunological events: alteration of lymphocyte subset toward CD4 cells, polyclonal activation of B cells, upregulation of major histocompatibility complex (MHC) class II and costimulatory molecules like B7, CD40 and ICAM-1, enhancing the levels of IL-2Rα and induction of Th2 cytokine response [27]. When used as a carrier molecule for heterologous antigenic determinants, CTB is capable of inducing high serum antibody titers in addition to secretory IgA. These features of CTB were taken into account when we were looking for a way to enhance the immune response to the hypervariable region 1 of the HCV envelope 2 protein.

The role of HVR1 in the development of a protective immune response against HCV is now being questioned [1] and the importance of a T-cell-based response in viral clearance is under intensive investigation [14]. However, MAbs, specific for aa 384–391 within HVR1 interfere with the interaction of HCV E2 glycoprotein with CD81 [11] which is the putative HCV cellular receptor [18] suggesting a functional role of HVR1 in viral attachment and entry into the host cells. Hence, HVR1 may be a prominent target for host neutralizing activity. The latter observation has been postulated and experimentally shown to be true [25, 28, 29] including an experimental vaccine in chimpanzees [9]. If the capacity of HCV to consistently escape immune recognition is indeed related to HVR1 heterogeneity, an HVR1-consensus approach developed by Puntoriero et al. [19] is an attractive strategy for obtaining cross-neutralizing HCV antibodies.

Besides its possible value as a vaccine candidate, cross-reactivity of plant-produced CTB-displayed HVR1 epitope with different HCV genotypes could potentially make this chimeric protein a simple and inexpensive diagnostic tool for the virus. In addition, the fact that the CTB component of the chimeric antigen induced high anti-CTB IgG titers itself actually suggests another application of this “multiple” plant-synthesized vaccine candidate.

It has been recently demonstrated that intranasal immunization with a plant virus-derived antigen stimulates better mucosal and systemic immune response than oral administration [7]. In this context, intranasal delivery of CTB/HVR1 by drops or spray would be more beneficiary and would require lower doses to elicit antibodies. In addition, research on the production of a CTB/HVR1 edible vaccine in transgenic food plants for oral administration is presently in progress in our laboratory.

The industrial scale production of biopharmaceuticals in plants is clearly a matter of the near future [4, 12]. Working strategies for plant vaccines against individual pathogens as developed in our study and by other researchers will

then be available for immediate practical use. With no efficient natural vector to transmit the virus in the field, recombinant TMV does not represent a biohazard; furthermore, the "foreign sequence" will not remain in the environment after production because of the subsequent reversion of the recombinant virus to the wild type TMV [12, 26].

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