Humoral Immune Response against a Hepatitis C Virus Envelope E2 Variant Expressed in Escherichia coli

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ABSTRACT

A hepatitis C virus (HCV) E2 variant encompassing amino acids 458 to 680 was expressed in *Escherichia coli*. The recombinant product (His-E2$_{680}$) was immunoidentified as a 30-kD protein using the serum from a patient with chronic hepatitis C, and was purified using Ni^{2+}-NTA chelate chromatography. When HCV-positive human sera were tested with the His-E2$_{680}$ protein, 39% of them showed anti-E2 reactivity. ELISA experiments showed that this antigen was very immunogenic in immunized rabbits. Using synthetic peptides, we identified an immunologically relevant region in the 643–671 segment of the His-E2$_{680}$ protein.

Keywords: E2 protein, *E. coli* expression, humoral immune response, HCV

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RESUMEN

Respuesta inmune humoral contra una variante de la proteína E2 del virus de la hepatitis C expresada en *Escherichia coli*. Se expresó en *Escherichia coli* una variante de la proteína E2 del virus de la hepatitis C (VHC), que comprende los aminoácidos entre 458 y 680. El producto recombinante (His-E2$_{680}$) fue immunoidentificado como una proteína de 30 kD con un suero de un paciente infectado crónicamente por el VHC, y fue purificado mediante cromatografía de afinidad por iones metálicos (Ni^{2+}-NTA). La proteína His-E2$_{680}$ mostró 39% de reactividad frente a un panel de sueros humanos positivos para el VHC. Mediante un ELISA se demostró la alta inmunogenicidad de este antígeno en conejos, y mediante la utilización de péptidos sintéticos identificamos una región inmunodominante comprendida entre los aminoácidos 643 y 671 de la proteína His-E2$_{680}$.

Palabras claves: expresión en *E. coli,* proteína E2, respuesta inmune humoral, VHC

*I*ntroduction

The hepatitis C virus (HCV) is the major etiological agent of post-transfusional non-A, non-B hepatitis throughout the world [1] and is considered as one of the causative agents of chronic hepatitis, cirrhosis and hepatocellular carcinoma. So far, the only available treatment for chronic HCV infection is through α-IFN therapy although its long-term effect occurs only in 10–30% of patients [2]. Therefore, the development of a vaccine to prevent HCV infection is highly sought.

The virus has a positive-strand RNA of about 9500 nucleotides with a single open reading frame encoding a precursor polyprotein of about 3000 amino acid residues. The precursor polyprotein is processed into the core (C), envelope-1 (E1), envelope-2 (E2) and several nonstructural proteins by the host signal peptidase and the viral proteinase [3, 4].

Evidence from clinical and experimental studies on human and chimpanzees suggests that the HCV envelope glycoprotein E2 is a key antigen for developing a vaccine against HCV infection [5–8]. The viral envelope glycoprotein E2 has been suggested to be responsible for binding of the virus to the target cell, and the antibodies to this region have been proposed to neutralize the virus and to drive immune selection. Several observations suggest that the hypervariable region 1 (HVR-1), located at the N-terminus of E2 [9–11], contain cytotoxic T-lymphocyte and B-cell linear epitopes [12, 13]. However, the higher genetic variability of this region may allow the virus to escape immune surveillance, and it represents a serious problem in the development of a broadly reactive vaccine against HCV infection [14–17]. Nevertheless, certain domain of biological importance within HCV E2 sequences must be preserved. It is very important for the development of a HCV vaccine to determine which region of E2 is critical for binding to the host cell receptor, and to identify the genotype-conserved determinants [18, 19]. Recently, it was also reported the existence of B-cell epitopes within the E2 protein downstream from HVR-1 [18, 20–22] and that the neutralizing epitope(s) in HCV E2 protein may also be outside HVR-1 [23].

In this work, we expressed an HCV E2 variant (His-E2$_{680}$) in *Escherichia coli* that does not include the HVR-1 region. We investigated the humoral immune response to this antigen. To this aim, we analyzed the reactivity of His-E2$_{680}$ protein in immunized rabbits and also evaluated the response against three synthetic peptides that cover different regions of the His-E2$_{680}$ protein. In addition, the recognition pattern of the His-E2$_{680}$ protein with a panel of HCV-positive human sera was also characterized.

*M*aterials and Methods

Cloning, expression and purification

A DNA fragment encoding amino acids (aa) 458 to 680 of the HCV E2 protein of the viral polyprotein was obtained by polymerase chain reaction (PCR) from pRE-5 (a plasmid containing the structural region of a

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Cuban HCV strain) [24]. For amplification we used as primers the oligonucleotides: 5-GATGGATCCGCCAGCTGCCGCCATTGAT-3, which contain a *Bam*HI site, and 5-AGCCAAGC TTCGTTAGAAGGAGCAGGGCAGTAT-3 which contain a *Hind*III site. The amplified segment was cloned *Hind*III–*Bam*HI into the plasmid pRSET-A (Invitrogen, USA). This plasmid contains a hexa-histidine tag upstream of the multiple-cloning site under the control of the T7 promoter. For expression of the E2 variant, *E. coli* BL21 (DE3) strain was transformed with the pRSETAE2(680) expression vector. The production of His-$E2_{680}$ was induced with 0.5 mM IPTG following the procedure previously described [25]. After cell disruption using a French press at a pressure of 100 bar, the insoluble fraction was washed with 0.01 M Tris containing 2 M urea, 1% Triton X-100, pH 8.0, and solubilized with urea buffer (8 M urea, 0.1 M NaH_2PO_4, pH 8.0). The supernatant was loaded onto a pre-equilibrated Ni^{2+}-NTA column (Quiagen, Chatsworth, CA). After washing the column with pH 8 and 6.3 respectively, the proteins were eluted with urea buffer pH 4.3. The renaturation procedure was performed by a stepwise, ten-fold dilution using the same buffer without the chaotropic agent and containing 3 g/L PEG 4000. The final yield of the process was determined by comparing the protein band with a carboanhidrase standard on Coomassie-blue stained gels, following the method of Bradford [26].

SDS-PAGE and Western blot

Samples from cellular extract and purified His-$E2_{680}$ were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [27], and were either stained with Coomassie brilliant blue R250 (Sigma, USA) or electrotransferred [28] to a Hybond-C membrane (Amersham, UK). The amount of expressed protein was estimated from a stained gel using a densitometer (CS-9000, Shimadzu, Kyoto, Japan). The transferred sheet was treated for 1 h at room temperature with phosphate-buffered saline containing Tween-20 0.05% (PBS-T) and 5% skim milk. The membrane was incubated during 1 h at 37 ºC with a 1:10 dilution of patient serum free from specific antibodies to *E. coli* proteins [29]. After washing, the membrane was allowed to react with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-human IgG (Amersham, UK) for 1 h at 37 ºC. Immunoreactivity was detected using 0.2 mg/mL 9-amino-4-ethyl-carbazole (Sigma, St. Louis, USA) and 0.05% (v/v) H_2O_2 (Caledon, Canada).

Immunization protocol

Two 4-months-old female rabbits were subcutaneously immunized with 100 µg of His-$E2_{680}$ antigen emulsified in complete Freund's adjuvant. After primary immunization the rabbits were boosted at 3, 6 and 9 weeks in the same antigen emulsified with incomplete Freund's adjuvant. Serum samples were taken at 0, 2, 5, 8 and 11 weeks.

Peptides

The following synthetic peptides covering different regions of the HCV E2 protein were used to evaluate the reactivity of the sera from His-$E2_{680}$-immunized rabbit:

e2.1 (544–569): PPQGNWFGCTWMNSTGFT KTCGGPPC

e2.2 (643–671): ACNWTRGERCDLEDRDRSL ESPLLLSTTE

e2.3 (657–683): DRDRSELSPLLLSTTEWQI LPCSFTTL

e2.4 (421–447): FNASGCPERMASCRPIDEF AQGWGPIT

Peptides were synthesized according to the solid-phase method [30]. All peptides were purified by reverse-phase high performance liquid chromatography (RP-HPLC) (Vydac C18, 10 x 250 mm). Peptides were solubilized in dimethyl sulfoxide/H20 (1:24, v/v) at a concentration of 2 mg/mL.

Human sera

A panel of human sera (UMELISA HCV, Centro de Inmunoensayo, Havana, Cuba) from 280 anti-HCV positive individuals was obtained from a Blood Donor Unit and Liver Units from five hospitals in Havana City. Among them, 55 were HCV-positive blood donors, 51 characterized as chronically infected individuals and 60 as non-chronically infected. The rest of the sera were not well characterized. Ten sera from normal non-infected blood donors were used as negative controls.

Enzyme-linked immunosorbent assay

The His-$E2_{680}$ protein was diluted to a concentration of 20 µg/mL and the synthetic peptides were diluted to 5 µg/mL in carbonate-bicarbonate coating buffer. Microtiter plates (High Binding Costar, Cambridge, MA) were coated with 100 µL of antigen overnight at 4 ºC. After washing three times with PBS-T the plates were incubated with patient sera (1:10 dilution in 2% skim milk in PBS-T) or sera from immunized rabbit (in dilutions ranging from 1:10 to 1:160,000), for 1 h at 37 ºC. The plates were washed three times and then incubated with a 1:40,000 goat anti-human (or rabbit) IgG sera conjugated to horseradish peroxidase (Amersham, UK), for 1 h at 37 ºC. The plates were then washed three times with PBS-T and incubated at room temperature for 10 min, with 100 µL of a solution containing 0.05% (w/v) *o*-phenylenediamine (Sigma, St. Louis, USA) and 0.05% (v/v) H_2O_2. Subsequently, 50 µL of 3 N H_2SO_4 were added to stop the reaction, and the absorbance at 492 nm was determined in an ELISA reader (SensIdent Scan, Merck). The cutoff value was established by duplication of the mean absorbance value of rabbit pre-immune serum and normal human serum from three determinations.

Results

Expression and purification of His-$E2_{680}$

The His-$E2_{680}$ protein was produced by a recombinant *E. coli* strain. This E2 protein lacks the hydrophobic C-terminal domain and contains a hexa-histidine tag. In addition, the His-$E2_{680}$ protein lacks the HVR-1 located at the amino terminus. BL21 (DE3) competent cells were transfected with the expression plasmid. Cellular extracts of transformed or non-transformed bacteria were subjected to SDS-PAGE (Figure 1A). After adding IPTG at 0.5 mM, a prominent band of an apparent molecular weight of 30 kD, that represents about 20%

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of the total cellular proteins, was observed. After cell disruption we observed that most of the His-E2$_{680}$ protein was in the insoluble fraction (data not shown). Western blot analysis using serum from an HCV-infected patient (free from specific antibodies to *E. coli* proteins) showed the specific recognition of His-E2$_{680}$ protein (Figure 1B). The E2 protein was purified to 90% under denaturing conditions by Ni^{2+}-NTA chromatography, as shown in Figure 1A, lane 3. The final yield was approximately 0.8 mg/L of initial *E. coli* culture, as determined by the method of Bradford [26].

Characterization of the humoral response induced by His-E2$_{680}$ protein in rabbits

Polyclonal antisera were raised in two female rabbits by immunizing with purified His-E2$_{680}$ antigen. Rabbits developed high levels of antibodies against the His-E2$_{680}$ protein. The time course of the antibody response to His-E2$_{680}$ antigen is shown in Figure 2. Rabbits seroconverted two weeks after the primary immunization. Maximal titers (1:160,000) were found 15 days after the final booster.

Rabbit sera were tested against three synthetic peptides covering different regions of the E2 variant. As shown in Figure 3, both sera recognized the three synthetic peptides, being the e2.2 peptide (aa 643–671) the one recognized with higher intensity. The titers against this peptide were about eight-fold higher than the others.

Reactivity of His-E2$_{680}$ protein against HCV-positive human sera

We evaluated the ability of His-E2$_{680}$ antigen to react with human sera in an ELISA. Two-hundred eighty sera positive by UMELISA anti-HCV were screened with a protein derived from His-E2$_{680}$ *E. coli* and 109 of them were positive. This study shows that anti-His-E2$_{680}$ antibodies are more frequently detected in hepatitis C chronic patient's sera than in non-chronic (Table).

*D*iscussion

HCV proliferates very poorly in humans and chimpanzees—the only experimental animal susceptible to HCV infection [31]—and proliferates inefficiently in cultured cell lines. Thus, most biochemical analysis of HCV proteins has been centered on either transient or stable expression in cells using plasmids or virus expression vectors. Characterization of the HCV envelope proteins may provide insight into the understanding of the mechanism of HCV persistence and may also lead to the possible development of an HCV vaccine.

Some studies strongly suggest that neutralization of HCV would mostly be type-specific, involving the participation of variable, non-conserved epitopes [32, 33]. Nonetheless, recent observations have suggested the existence of other neutralization determinants, cross-reactive and not directed at the HVR-1 [34]. Studies by Rosa *et al.* [23] and François *et al.* [35] indicated that the determinant(s) recognized by NOB (neutralizing of binding) antibodies are likely directed at conformation-dependent domains of E2, which appear to

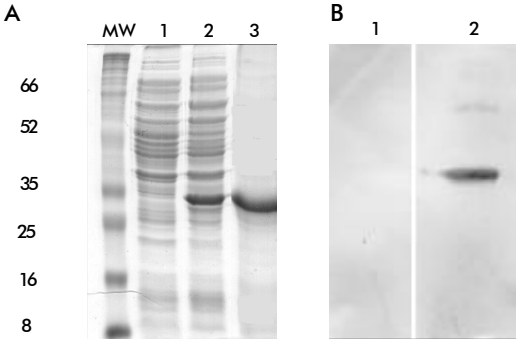

Figure 1. SDS-PAGE (A) and Western blot (B) of *E. coli* BL21 (DE3) cellular extract showing expression of a recombinant E2 protein variant. Lane 1 (A and B), non-transformed BL21 (DE3) strain; lane 2 (A and B), BL21 (DE3) strain transformed with pSETAE2 (680) and induced with IPTG; lane 3, purified His-E2$_{680}$ protein after Ni^{2+}-NTA chromatography. Western blot pattern was obtained by using anti-HCV-positive human sera (1:10 dilution). MW, molecular weight marker (kD).

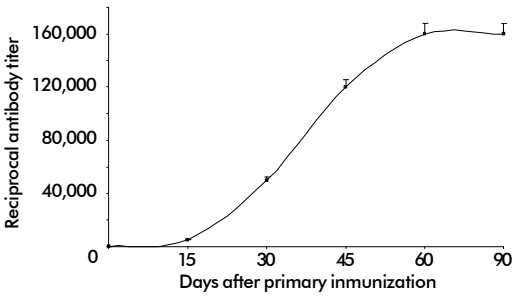

Figure 2. Time course of antibody production in sera of rabbits immunized with the recombinant E2 protein. Data represent the titer (average standard deviation of values from two rabbits) obtained in an ELISA against His-E2$_{680}$ antigen. Rabbits were boosted at 30, 45 and 60 days after the primary immunization. Serum samples were taken at two week intervals.

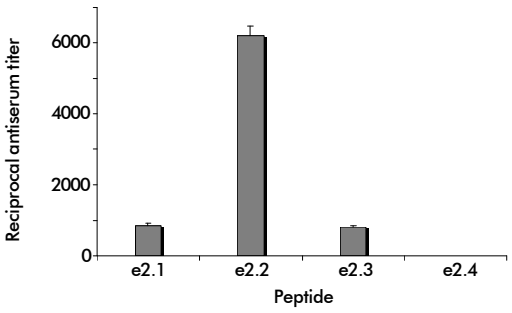

Figure 3. Recognition of synthetic peptides by rabbit His-E2$_{680}$ specific sera. Results represent the mean titer (± standard deviation) of sera from two rabbits individually tested against each peptide. Another synthetic peptide (e2.4) outside 458–680 E2 variant was used as negative control.

Table. Anti-His-E2680 reactivity studied in HCV-positive patients.

Serum sample	Total	Anti-His-E2$_{680}$ positive (%)
Anti-HCV positive	280	109 (39)
Chronic	51	21 (41)
Non-chronic	60	10 (16)
Blood donors	55	10 (18)
Healthy human	10	0 (0)

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be conserved among different genotypes and seem to be out of HVR-1.

In our work, we efficiently expressed an E2 fragment (aa 458–680) in *E. coli*. The HVR-1 was not included in our recombinant, N- and C-terminal truncated protein. The C-terminal hydrophobic region of E2 protein might be toxic for *E. coli* growth [36]. Another variant of HCV-E2 protein (aa 384–650) fused to C-Lyt binding domain has been expressed to lower levels in *E. coli* W3110 strain using the lpp/lac promoter system [25].

His-$E2_{680}$ was immunoidentified by Western blot analysis as a 30-kD protein, using serum from a chronically infected patient. After cell disruption, this protein was found to be associated to the insoluble fraction. His-$E2_{680}$ was obtained with 90% purity after the purification procedure. The final yields of purification after the denaturing purification procedure were according to a previous report using a similar expression system [37].

Recombinant His-$E2_{680}$ protein induced high levels of antibodies when rabbits were immunized. Although it was obtained in a different animal species; this result is in agreement with previous works that have reported recombinant E2 variants that elicited strong humoral immune responses [25, 38].

The His-$E2_{680}$ protein was recognized by 39% of the human infected sera. The recognition frequency was higher in chronic patients than in non-chronic and blood donors, which confirmed previous findings of envelope antibodies during viraemia. Due to the persistent liver injury in chronic patients, many nonmature (denatured or non-glycosylated) forms of the E2 glycoprotein have been exposed to the immune system for a long time, whereas non-chronic patients and blood donors have a shorter period of disease evolution with little or no liver injury. The frequency of antibodies was lower than previously observed for another variant of *E. coli*-derived E2 protein (58% of anti-HCV-positive persons) [39]. The same reports show a seroprevalence of > 90% of antibodies directed at the E2 protein among patients with chronic HCV infection, when a glycosylated recombinant construct of E2 was used as a solid-phase antigen. This would indicate that a correct tertiary structure and glycosylation is of high importance for the recognition of the HCV-E2 protein by HCV-infected patients.

In order to identify B-cell epitopes in our recombinant proteins, three synthetic peptides were selected for this preliminary study, because they have been reported to contain E2 immunoreactive epitopes [40, 41]. Previous studies with chronic HCV-infected patients using synthetic peptides showed no reactivity with HCV-E2 (aa 643–671) epitopes [42]. In our work, we identified the linear region (aa 643–671) as the most immunodominant within HCV-$E2_{680}$ protein for immunized rabbit sera.

Antibodies against HVR-1 have been reported from rabbits immunized with synthetic peptides, which neutralized HCV *in vitro* [43] and *in vivo* [32]. However, the isolate-specific function of these anti-HVR-1 antibodies is a major impediment to the development of effective vaccines. Two regions of the E2 protein downstream from HVR-1, aa 464–503 and aa 514–543, were immunoreactive in an ELISA using peptides and may be involved in the cross-recognition of virus capture [34].

In this work, we demonstrated that an active immune response against the His-$E2_{680}$ protein was induced in rabbits. Moreover, we identified a novel immunologically relevant region (aa 643–671) in the immunized rabbits that has never been reported before as immunoreactive in humans with natural HCV infection.

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