# Antigenicity and immunogenicity of the hepatitis C virus envelope E2 protein

✍ Gillian Martínez-Donato, Santiago Dueñas-Carrera, Liz Alvarez-Lajonchere, Juan Morales, Nelson Acosta-Rivero, Eduardo Martínez, Ariel Viña, Ivis Guerra, Angel Pérez, Alexis Musacchio, José García, Osvaldo Reyes, Hilda E Garay, Luis J González, Julio C Alvarez, Yordanka Soria

Center for Genetic Engineering and Biotechnology
PO Box 6162, Havana 10600, Cuba
E-mail: gillian.martinez@cigb.edu.cu

**REPORT**

**ABSTRACT**

The absence of an available animal model or a consistent *in vitro* culture system for the replication of the hepatitis C virus (HCV), have seriously limited the research on the immunological role of their main antigens. In the present paper, four HCV E2 protein variants were obtained from recombinant microorganisms. Particularly, the E2.680 protein (384-680 aa) constitutes the first E2 protein variant obtained from recombinant *Picchia pastoris*. Moreover, peptides covering different regions of the HCV E2 protein were synthesized. The recombinant proteins and synthetic peptides were used to study the recognition of HCV infected human sera. As a result, we describe for the first time the existence of an immunologically relevant region covering not only the HVR-I but also the region up to 473 aa in the HCV polyprotein. Furthermore, it was demonstrated that the E2.680 protein was recognized by a specific monoclonal antibody for conformational epitopes. This is the first evidence of an E2 variant obtained from recombinant microorganisms containing regions that are conformationally similar to the viral antigen. The recombinant protein variants and the synthetic peptide of the HVR-I, were able to induce high titers of specific antibodies in animal models, but the E2.680 variant additionally induces a specific cellular immune response. We conclude that, the N-terminal region of the HCV E2 protein plays a main role in antigenicity and viral immunogenicity. Therefore, the E2.680 protein and the HVR-I synthetic peptide obtained are available analytical tools and can potentially become components of a vaccine candidate against HCV.

## Introduction

At present, there are more than 170 million people infected by HCV in the world [1]. In Cuba, it has been reported that approximately 0. 8 % of the population has antibodies against this pathogen [2]. This virus produces a persistent infection in more than 85% of the cases, leading to chronic hepatitis. Approximately 20% of the chronic cases lead to cirrhosis that can degenerate in hepatocellular carcinoma [3]. Currently, the best alternatives for the treatment against HCV are generally effective in only 55% of the cases [4]. For this reason, the generation of an effective vaccine against this pathogen would have, both nationally and internationally, an important economic and social impact. The absence of an *in vitro* culture system for the assembly and release of viral particles has limited the knowledge on the maturation of glycoproteins and viral morphogenesis. Although the mechanism by which the virus enters the cell is unknown, it is thought that the E2 envelope glycoprotein plays a major role at this stage of the viral cycle [5]. In a chimpanzee model study, *in vivo* protection was achieved by vaccinating with recombinant HCV E1/E2 proteins, and the anti-E2 antibody titers were correlated with protection [6]. Antibodies found in the sera of patients could prevent infection when incubated *in vitro* with the virus before infecting the chimpanzee [7]. Moreover, HCV E2 proteins expressed in Chinese hamster ovary (CHO) cells bound with high affinity to human cells, and sera from protected chimpanzees, contained antibodies which neutralized the binding of the E2 protein to target cells [5]. Therefore, the E2 protein is a target of interest for the development of a vaccine or an effective antiviral treatment against HCV.

The present work is based on the hypothesis that it is possible to obtain E2 protein fragments from recombinant microorganisms or through chemical synthesis that may be useful for generating relevant information on the antigenicity and immunogenicity of the HCV E2 protein.

## Materials and methods

### Proteins and Peptides: synthesis, purification and characterization

The proteins E2C, E2A, Clyt-E2: HCV E2 encompassing 382 to 605, 458-650, 384-650 amino acids of the viral polyprotein, respectively, and expressed in *E. coli* cells, have been previously described [8, 9].

The E2.680: HCV E2 protein encompassing 384 to 680 aa of the viral polyprotein, and expressed in *Pichia pastoris* cells was described in another paper [10], where we also described the HCVcore and HBsAg, used as a negative control in ELISA and a Delayed-type hypersensitivity assay.

The following synthetic peptides, covering different regions of the HCV E2 protein, were used here: P-5 (436-466): GFIAALFYAHRFNASGC-PERNASCRPIDEFA; P-9 (447-473): FNASGC-PERMASCRPIDEFAQGWGPIT; P-10 (420-446): HINRTALNCN DSLQTGFIAALFYAHR; P-12 (544-569): PPQGNWFGCTWMNSTGFTKTCGG-PPC; P-15 (384-414): TGTYVTGGTAARGVSQFT-GLFTSGPSQKIQL (encompassing the HVR-1). The numbers in parenthesis indicate the aa position on the viral polyprotein.

1. Kato N. Molecular virology of hepatitis C virus. Acta Med Okayama 2001;55(3): 133-59.

2. WHO. Health in the Americas. Edition PAHO Scientific and Technical Publication 2002;587:207-8.

3. Gotto J, Dusheiko GM. Hepatitis C and treatment with pegylated interferon and ribavirin. Int J Biochem Cell Biol Octuber 2004; 36(10):1874-7.

4. Fried MW, Shiffman ML, Rajender RK, Smith C, Marinos G, Gonzales FL, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J A, Yu J. Peginterferon α-2a plus rivabirin for chronic hepatitis C Virus infection. N Engl J Med September 2002;347(13):975-82

5. Rosa D, Campagnoli S, Moretto C, Guenzi E, Cousens L, Chin M, Dong C, Weiner A.J, Lau JYN, Choo Q-L, Chien D, Pileri P, Houghton M, Abrigniani S. A quantitative test to estimate neutralizing antibodies to the hepatitis C virus: cytofluorimetric assessment of envelope glycoprotein 2 binding to target cells. Proc. Natl. Acad. USA, 1996;93:1759-63.

6. Choo Q-L, Kuo G, Ralston R, Weiner A, Chien D, Nest GV, Han J, Berger K, Thudium K, Kuo C, Kansopon J, McFarland J, Tabrizi A, Ching K, Moss B, Cummins LB, Houghton M, Muchmore E. Vaccination of chimpanzees against infection by the hepatitis C virus. Proc. Natl. Acad. Sci. USA, 1994; 91:1294-8.

7. Farci P, Alter HJ, Wong DC, Millers RH, Govindarajan S, Engel R, Shapiro M, Purcell RH. Prevention of hepatitis C virus infection in chimpanzees after antibody-mediated in vitro neutralization. Proc. Natl. *Acad. Sci.* USA, 1994;91:7792-6.

✍ Author of correspondence

The peptides were synthesized using the Boc/Bzl strategy and the "tea bag method" [11] on 60 mg of MBHA resin (substitution level 1 mmol/g) each. All Boc-aa and the MBHA resin were obtained from Bachem (Switzerland). Solvents were obtained from Caledon (Canada) or Merck (Germany). The Boc group was used for $N^a$-protection. The side-chain protecting groups used were 4-methoxybenzyl (Mob) for Cys; Bzl for Ser, Asp, Glu and Thr; tosyl (Tos) for Arg; 2,4-dinitrophenyl (Dnp) for His; dichlorobenzyl ($Cl_2$-Bzl) for Tyr; formyl (For) for Trp and chlorobenzyloxycarbonyl (Cl-Z) for Lys. Cleavage of the Boc-group was carried out with 37.5% TFA in DCM for 30 min. The TFA salt was neutralized by 5% DIEA in DCM three times for 2 min each. The aa were coupled using DIPCDI. Side-chain deprotection and cleavage from the resin was performed following the "Low-High" HF procedure [13] with HF-DMS-EDT-$p$-cresol (25:60:10:5) for 2 h at 0 °C and with HF-DMS-anisole-thiocresol (79.8: 10: 10: 0.2) for 1 h at 0 °C, respectively. The peptides were extracted with 30% HAc in water and lyophilized.

Peptides were purified by HPLC (Pharmacia-LKB, Sweden), using a RP-C18 column (25 x 250 mm, Vydac). A linear acetonitrile/water gradient from 15 to 60% B for 60 min and a flow rate of 4 mL/min were used. The peptides were analyzed with a RP-C18 column (4.6 x 150 mm, Vydac) and a linear gradient from 5 to 60% B in 35 min, the flow rate was 0.8 mL/min. The absorbance was monitored at 226 nm.

Peptides were characterized by aa analysis on an automatic amino-acid analyzer Alpha Phus 4151 (Pharmacia-LKB, Sweden), using a sodium buffer system for amino-acid separation and ortho-phthalaldehyde derivatization for fluorescence detection. All samples were analyzed using two replicates.

### Human sera

Samples of human sera were obtained from the Centro de Inmunoensayo (Havana, Cuba) where they were previously screened for the presence of anti-HCV antibodies by UMELISA HCV (Centro de Inmunoensayo). Anti-HCV positive sera were also confirmed by Ortho HCV 2.0 ELISA (Ortho Diagnostic Systems, USA).

### Immunization schedule

BALB/c female mice, 6 to 8 weeks old (18-20 g of weight), were purchased from CENPALAB (Ciudad de La Habana, Cuba) and used for all in vivo studies. Groups of 10 mice were intramuscularly injected (im) into the quadriceps muscle with either 0, 1, 10 or 20 µg of E2680 or with 1, 10 or 20 µg of HBsAg at weeks 0, 2 and 4. Protein was administered at a final volume of 100 µL, adjuvanted in Aluminium Hydroxide (AL) (0.5 mg Al3+ for 20 µg of E2 or HBsAg). Blood samples were collected at weeks 1, 3 and 5, after primary immunization from the retro-orbital sinus and the sera were analyzed for antibodies (Ab) against the HCV E2 protein. Five animals per group were either slaughtered after the final blood extraction or used for delayed- type hypersensitivity (DTH) studies.

### *Enzyme-linked immunosorbent assay (ELISA)*

The 96-well microtiter plates (Costar, Cambridge, MA) were coated with 100 µl of E2 or c-(HCVcore) and HBsAg (as negative controls) proteins (5 µg/mL) or synthetic peptides (1 µg/mL) diluted in coating buffer (50 mM carbonate buffer, pH 9.6) at 4 °C overnight. After three washes with PBS containing 0.05% Tween 20 (PBST), the wells were blocked with 200 µl of PBST containing 1% skim milk at room temperature for 1 h. Each well received 100 µl of human (1:20 diluted) or mice (in dilutions ranging 1:10 to 1:120 000) serum in PBST, the monoclonal antibodies were diluted 1:100 and the plates were incubated at 37 °C for 1 h. The plates were washed five times with PBST, and 100 µl of 1: 3000 diluted horseradish peroxidase-conjugated goat anti-human (or mouse) IgG (Amersham, UK) were added. The plates were incubated at 37 °C for 1 h, washed again five times with PBST and incubated at room temperature for 10 minutes 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 a SensIdent Scan (Merck, Germany). The cutoff value was double the mean absorbance value of mouse pre-immune sera (or human anti-HCV negative sera) from three determinations.

### Delayed-type hypersensitivity assay (DTH)

DTH response induced by E2.680 was assayed by the footpad-swelling test 21 days after the third immunization [12]. For the test, mice were injected with 5 mg of recombinant truncated E2680 into the right footpads. As a negative control, HBsAg was injected into the left footpads. The swelling response was measured 72 h after the injection.

### Statistical analysis

To compare differences among groups, a One-way ANOVA with the Newman-Keuls post-test or Kruskal-Wallis non-parametric statistic test with Dunn's Multiple Comparison Test as post-test were used. $P < 0.05$ was considered significant.

## *R*esults

### Recognition of E2 synthetic peptides by mouse immune sera

The reactivity of anti-E2 antibodies in mice immunized against five synthetic peptides, covering different regions of the E2 protein, was studied to identify the B-cell epitope regions in our recombinant proteins. Specific anti-d-E2C mouse sera (1:200 diluted) reacted with P-5, P-12 and P-15 peptides. However, mouse polyclonal antibodies induced by nd-E2C only reacted at a similar dilution with P-5 and P-15. Anti-E2A mouse sera (1:200 dilution) exclusively recognized the P-12 peptide. No reactivity was found against P-9 and P-10 peptides (Figure 1).

### Reactivity of HCV+ human sera against the nd-E2 protein and E2 synthetic peptides

We evaluated the ability of the nd-E2C antigen and the E2 synthetic peptides to react with human sera in ELISA. No specific recognition was detected with thirty-three sera that were negative for anti-HCV antibodies. In contrast, 11 out of 39 sera from HCV

8. Dueñas-Carrera S, Viña A, Garay HE, Reyes O, Alvarez-Lajonchere L, Guerra I, González LJ, Morales J. Immunological evaluation of Escherichia coli-derived hepatitis C virus second envelope protein (E2) variants. J Peptide Res 2001;58:221-8.

9. Martínez-Donato G, Viña A, García JL, Morales J. Cloning and purification of Hepatitis C Virus E2 Protein fused to Choline-binding domain: Evaluation of humoral immune response in immunized rabbits. Biotecnología Aplicada 2000; 17:85-88.

10.Martínez-Donato G, Capdesuñer Y, Acosta-Rivero N, Rodríguez A, Morales-Grillo J, Martínez E, González M, Alvarez-Obregon JC, and Duenas-Carrera S. Multimeric Hepatitis C virus E2 protein obtained from Pichia pastoris cells induces a strong immune response in mice. Acepted in American Journal of Biochemistry and Biotechnology 2005.

11.Houghten RA, DeGraw ST, Bray MK, Hoffmann SR, Frizzell ND. Simultaneous Multiple Peptide Synthesis: The Rapid Preparation of Large Numbers of Discrete Peptides for Biological, Immunological, and Methodological Studies. Biotechniques 1986;4:522-6.

12. Kundig TM, Bachmann MF, Oehen S, Hoffmann UW, Simard JJ, Kalberer CP, Pircher H, Ohash PS, Hengartner H, Zinkernagel RM. On the role of antigen in maintaining cytotoxic T-cell memory. Proc. Natl. Acad. Sci. USA, 1996;93:9716-23.

13. Alvarez-Lajonchere L, Martínez-Donato G, Morales J, Aguilar JC, Dueñas-Carrera S. Immunization with HCV synthetic peptides conjugated to the P64k protein elicited strong antibody response in mice. Biotecnología Aplicada 2003; 20:209-13.
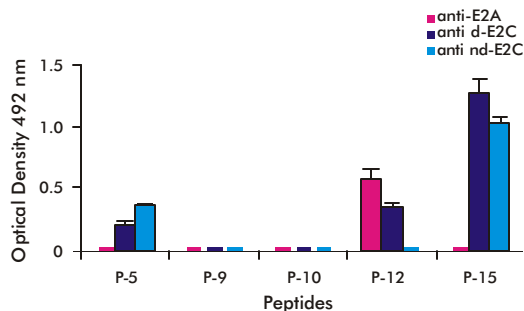
Figure 1. Evaluation of recognition by mouse anti-E2 sera (1:200 dilution), induced by E2A and both denatured and non-denatured E2C, of five synthetic peptides covering different regions of the E2 protein. Results represent the mean optical density ± standard deviation of sera from eight mice individually tested against each peptide. The cut-off value (double the pre-immune sera OD492nm average) was subtracted.
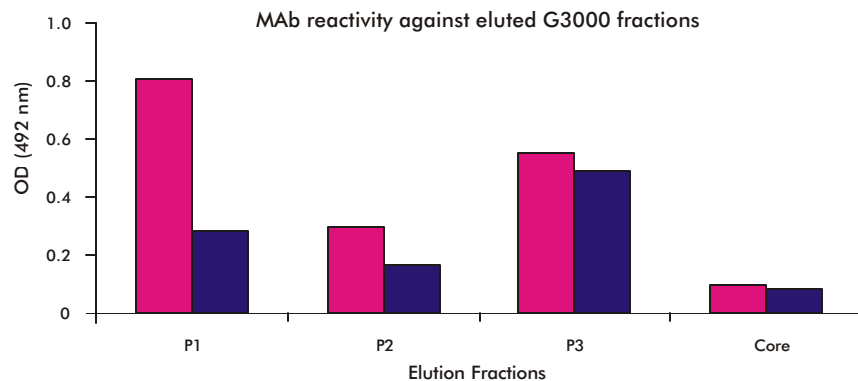


Figure 3. Reactivity of H52 and H53 MAbs against different oligomeric forms of the E2.680 protein (purified by molecular exclusion chromatography G3000). P1: represents the oligomeric form, P2 and P3 fractions oligomeric, trimeric and monomeric by ELISA assay.
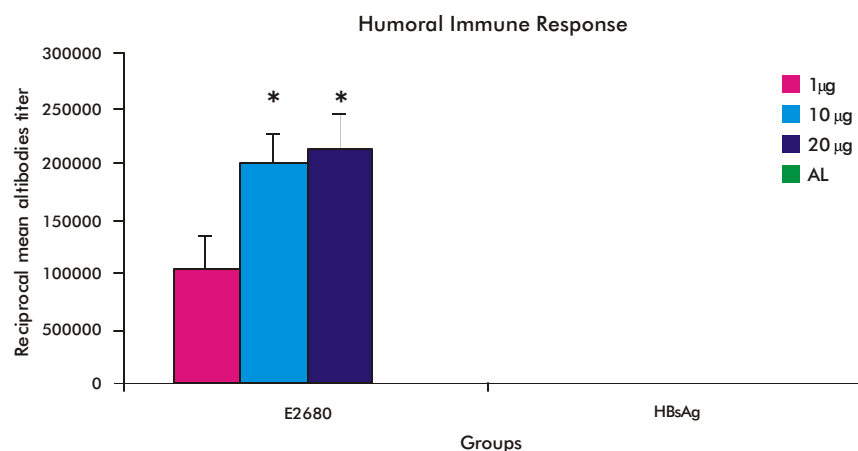


Figure 4. Induction of Anti-E2.680 antibody in mice. (Doses of 0 (AL), 1, 10 and 20 mg of either $E2_{680}$ or HBsAg were inoculated in mice). Results are shown as the reciprocal mean antibody titer. For clarity, error bars indicating the standard deviations of the means are shown in the positive sense only. (* $p < 0.01$ using Kruskal-Wallis non-parametric statistic test with Dunn's Multiple Comparison Test as post-test).

positive individuals (28.2 %), were reactive against the nd-E2C protein (data not shown). Nine of these eleven anti-E2 positive human sera (23.1 %) were also reactive to the P-15 peptide (Figure 2); the other synthetic peptides were unreactive to all tested human sera (data not shown).

## Antigenicity and immunogenicity of E2.680

To investigate the recognition of E2.680 by these MAbs, E2.680 was applied to a size exclusion chromatography G3000 column. Three fractions which possibly represent monomeric and different oligomeric forms of E2.680, according to the retention time of molecular weight markers were identified (Figure 3). All fractions containing E2.680 were recognized by H52 (Figure 3). However, fractions P1 and P3 (corresponding to the monomeric and trimeric fraction) but not P2, were recognized by the H53 MAb.

It is interesting to note that E2.680 was also specifically recognized by 70% of 31 anti-HCV positive sera (not shown).

As shown in Figure 4, E2.680 induced a potent humoral immune response in mice. Specific anti-E2.680 antibody titers above 1:200000 were detected in animals

immunized with 10 or 20 g of antigen. These titers were higher than those observed in animals immunized with 1 mg of antigen (titers of 1:100000) ($p < 0.01$).
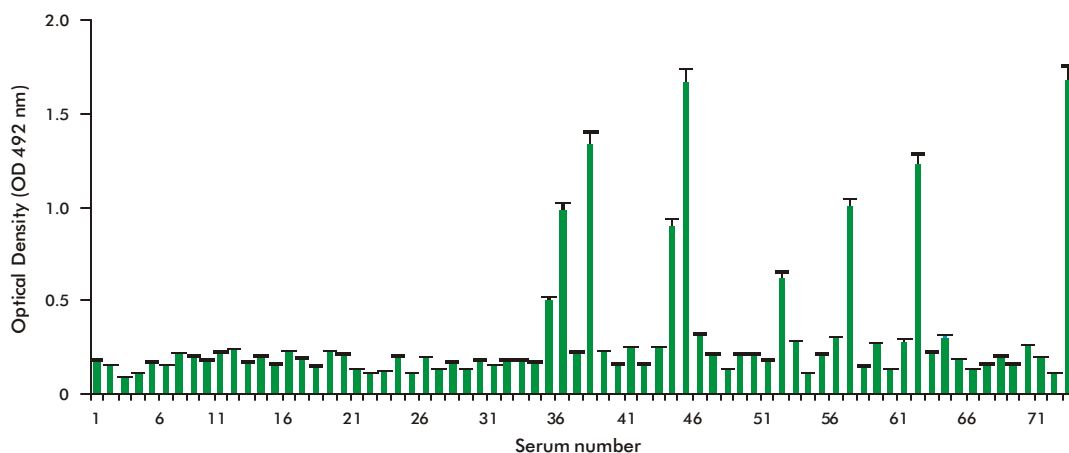


Figure 2. Evaluation of human sera from normal and anti-HCV positive individuals against P-15 peptide. From left to right, sera 1-33 were negative for anti-HCV antibodies; the rest were anti-HCV positive human sera. Results represent the average of three experiments ± standard deviation. For clarity, error bars are indicated in the positive sense only.

Finally, the *in vivo* anti-E2 specific cellular immune response was analyzed by DTH. As shown in Figure 5, the mice immunized with 1 and 10 mg of E2.680 showed a strong footpad swelling reaction. This response was higher than that of mice immunized with 20 mg of the antigen (p < 0.01).

## **D**iscussion

Natural HCV structural proteins are not useful for developing vaccines or specific anti-sera because the virus concentrations in the infectious materials are very low. Therefore, recombinant HCV structural proteins are helpful as immunogens. The characterization of the immune response generated against the antigens of the HCV and the parameters that correlate with the protection and clarification of the infection are not completely defined. For the development of preventive vaccines and therapeutic treatments against HCV, the E2 protein might be a crucial element and the results obtained in this work may therefore contribute to this effort.

We studied the reactivity of the recombinant proteins and the synthetic peptides before a panel of HCV infected human sera. The evaluation of 72 human sera, 33 anti-HCV negative and 39 positive sera, against E2A and E2C proteins demonstrated that 11 anti-HCV positive sera recognized E2C and 9 identified the P-15 peptide by ELISA. None of these human sera recognized the E2A protein or the rest of the synthetic peptides.
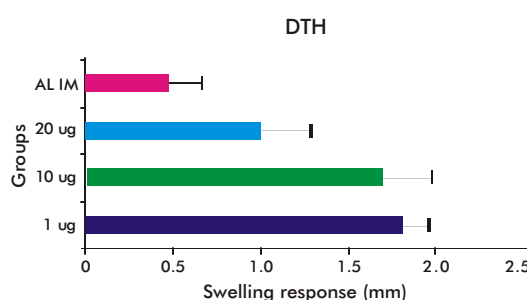


Figure 5. Induction of E2$_{680}$-specific DTH response in mice. The graph represents the average differences in swelling between the right and left footpads measured 72 h after injection. Standard deviation of three different determinations is represented by error bars in the positive sense only. (* mean statistical differences p < 0.01 using a one-way ANOVA test with the Newman-Keuls post-test).

The evaluation by ELISA of another panel of 31 anti-HCV positive human sera, this time against the E2.680 protein, revealed that 70% of these sera were recognized by this E2 variant produced by *Pichia pastoris*. Approximately 60% of the E2.680 positive sera had titers of between 1:100 and 1:200. None of the anti-HCV negative human sera recognized the E2.680 protein (data not/shown). These results together with those on the characterization of the recombinant protein variants identifical by mouse polyclonal antibodies against the HCV E2 protein [8, 9, 13] describe the existence of an immuno-dominant region at the B cell level in the E2 protein beyond the HVR-I, including the region from 384 until 473 aa in the viral polyprotein. The presence of a B cell epitope in the HVR-I and the possibility of inducing a strong antibody response against this region was demonstrated when it was conjugated to a carrier protein, but not when it was free, although these Abs did not recognize the HVR-I of other viral isolates [13].

On the other hand, the E2.680 protein is recognized by specific monoclonal Ab for linear (H52) and conformational (H53) epitopes, which indicates that this E2 variant contains regions similar to the viral antigen. In fact, the E2.680 protein obtained from the culture supernatant of *Picchia pastoris* is produced as a glycoprotein, modified with high mannose type oligosaccharides as demonstrated by endoglycosidase digestion with Endo H and PNGase F. It seems that this N-glycosylation pathway is partially homologous to the pathway in human cells [10].

The recombinant protein variants included here were also able to induce high titers of specific antibodies against the E2 protein when they were evaluated in BALB/c mice or rabbits, but the E2.680 variant, produced by *Pichia pastoris*, also induces a specific cellular immune response (Figures 1 and 2). The induction of specific cellular immune responses against Hepatitis C antigens are essential for the spontaneous resolution of acute Hepatitis C and long term protection.

According to these elements, the N-terminal region of the HCV E2 protein plays a main role in antigenicity and viral immunogenicity. Moreover, the recombinant protein variants and the synthetic peptides of the E2 protein, obtained in the present work, constitute valuable analytical tools and could be components of vaccine preparations against HCV.