Biochemical and Biophysical Research Communications **290,** 300–304 (2002)
doi:10.1006/bbrc.2001.6177, available online at http://www.idealibrary.com on IDEAL®

In Vitro Self-Assembled HCV Core Virus-like Particles Induce a Strong Antibody Immune Response in Sheep

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Received November 16, 2001

The *in vitro* self-assembly properties of the entire hepatitis C virus core protein (HCcAg) obtained from *Pichia pastoris* cells and the induction of specific antibody immune response were studied. HCcAg was purified as a low-molecular-weight species by electroelution under denaturing conditions for confirmation of its self-assembly properties. After renaturalization, electron microscopy showed that HCcAg assembled into spherical particles of 30 nm. HCcAg also showed homogeneity and was specifically recognized by serum from a chronic HCV carrier patient. The data indicated that *in vitro* assembly of HCcAg, into virus-like particles resembling HCV nucleocapsid particles at a mature stage, is an intrinsic quality of this protein. Finally, HCcAg generated a strong antibody immune response in sheep, suggesting its usefulness for stimulating the host immune response against HCV.

Key Words: **hepatitis C; core antigen; virus-like particles; *P. pastoris*.**

Hepatitis C virus (HCV) has been identified as a major causative agent for parenterally transmitted non-A, non-B hepatitis (1). At least 70% of infected patients develop chronic infection, and approximately 20% progress to cirrhosis (2). The genome of HCV is a single-stranded linear RNA of positive sense (3). It is approximately 9.5 kb in size and possesses an unique open reading frame, coding for a single polyprotein, flanked by noncoding regions at both its 5′ and 3′ ends (3, 4). The viral polyprotein is processed by a combination of host and viral proteinases into the mature viral proteins (5, 6). A core protein (HCcAg), at least two envelope proteins (E1 and E2), and a small putative protein of unknown function (p7) are encoded in the 5′ region of the open reading frame. At least six nonstructural proteins, including protease, helicase, and RNA polymerase enzymes and regulatory peptides are arrayed in the 3′ portion of the open reading frame. HCcAg, located at the amino terminus of the polyprotein, is highly basic in nature and is considered likely to be the viral capsid protein (6). The mature HCcAg is generated by cleavage at amino acid 173 (p21) (7). Since virion nucleocapsid formation involves multimerization of HCcAg and its interaction with viral RNA, these characteristics of HCcAg have been extensively studied and specific functions mapped to discrete portions of the molecule (8–10).

HCcAg could be attractive as an appropriate target for HCV vaccine strategies since it is highly conserved. Of the various regions of HCV, antibodies against HCcAg are detected most frequently in infected patients with HCV, and several B cell epitopes within the HCcAg have been determined (11). The association of T-helper (Th) cell response to the HCcAg with a benign clinical course has also been suggested (12). In addition, cytotoxic T-lymphocyte (CTL) epitopes have been identified in the HCcAg (13–15). The importance of the CTL response against noncytopathic virus for either viral clearance, or inmunopathogenesis is well established (16). Particulate or aggregate antigens have proven to be powerful immunogens able to produce not only a strong humoral immune response (17) but also a CTL response (18). We had previously shown that HCcAg expressed in *Pichia pastoris* cells, forms intracellular virus-like particles (VLPs) (19) resembling HCV nucleocapsid particles in a mature stage (20). In this study, the intrinsic capacity of HCcAg expressed in *P. pastoris* cells, to self-assemble *in vitro* into virus-like particles (VLPs) was studied. By evaluating the antibody immune response in sheep, HCcAg was found to be highly immunogenic. The VLPs containing HCcAg described here, could be useful not only for understand-

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0006-291X/02 $35.00

ing the intrinsic mechanisms involved in nucleocapsid assembly but also be a part of future therapeutic agents for anti-HCV treatment.

MATERIALS AND METHODS

Strains. The *P. pastoris* strain MP-36/C-E1.339, expressing the full-length HCcAg and the first 148 aa of the HCV E1 protein has been previously described (19). The MP-36 strain (19) was used as a negative control.

Antibodies. A mouse monoclonal antibody against the residues 5–35 of the HCV core protein (mAb SS-HepC.1) (20) was used for Western blot analysis. A human serum from a chronically HCV-infected patient (positive for HCcAg and negative for E1 antigen) (21) was used to detect HCcAg after gel-filtration chromatography analysis.

Growth conditions for P. pastoris strains. MP-36/C-E1.339 and MP-36 strains were grown using conditions already established (19). In brief, the MP36/CE1.339.5 was grown in minimal glycerol medium (MYG) (1.3% yeast nitrogen base, 1% glycerol, and 0.4 μg/ml biotin) at 30°C for 48 h. The HCcAg expression from the methanol oxidase promoter was induced by replacing MYG with minimal methanol medium (MM) (1.3% yeast nitrogen base; 0.5% methanol, 0.4 μg/ml biotin) and further incubation at 30°C for 96 h. The strain MP-36 was used as a negative control under the same growth conditions. At the end of the yeast cell culture, the cells were harvested and washed twice in TEN buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl).

Protein analysis. The protein samples were separated in a 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (22) and stained with Coomassie brilliant blue R-250 (CBB, Sigma, St. Louis, MO). The purity was determined using the software 1-D Manager for Windows 95 (version 2.0, TDI, SA, Madrid, Spain).

Immunoblotting assay. For immunoblotting, the samples were either resolved by SDS–PAGE and the proteins transferred electrophoretically to nitrocellulose membrane (HYBOND C, Amersham, England) or directly applied to nitrocellulose membrane. Binding of IgG antibodies was detected as previously described (23). In brief, the membrane was blocked for 1 h at room temperature with phosphate-buffered saline solution (PBS) (0.1 M NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4) containing 5% skim milk and then incubated either with mAb SS-HepC.1 or human serum for 1 h at 37°C. After washing, the membrane was reacted with 1/1000 dilution of horseradish peroxidase-conjugated protein-A (Amersham, UK) for 1 h at 37°C. Immunoreactivity was detected using a chemiluminescence-based detection kit (ECL, Amersham, England).

Purification of the HCcAg. Cells disruption was performed using glass beads in TEN buffer containing 6 M urea and 1 mM dithiothreitol. The suspension was diluted 10-fold in disruption buffer and incubated overnight at 4°C with gentle agitation. The lysate was clarified by centrifugation at 12,000*g* for 20 min. The extracted HCcAg was precipitated with 40% of ammonium sulfate at 4°C and was solubilized with sample buffer (50 mM Tris–hydrochloride, pH 6.8, 2.5% SDS, 5% (v/v) β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue). The sample was heated at 100°C for 30 min and centrifuged, and the supernatant was applied to SDS–PAGE (Bio-Rad, CA). The gel was negatively stained by incubating in 0.2 M imidazole solution for 20 min, followed by incubation in 0.2 M zinc sulfate solution. The gel was cut in the area corresponding to HCcAg molecular weight. HCcAg was then electroeluted in Tris–glycine SDS buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS). Electroeluted HCcAg (0.5 mg/ml) was renaturalized in the same electroelution system against Tris–glycine buffer (25 mM Tris base, 192 mM glycine). This sample was additionally dialyzed against TEN buffer containing 1 mM DTT and 1% glycerol (TENG). Renaturalized HCcAg sample was tested in three percent agarose gels prepared in 20 mM Tris–acetate–0.5 mM EDTA (TA) buffer and stained with TA containing 10 μg/ml of ethidium bromide.

Analysis by gel-filtration chromatography. One milliliter of either renaturalized HCcAg (0.1 mg/ml) or the particulate hepatitis B surface antigen (HBsAg) (24) (0.1 mg/ml) was applied to a TSK G5000 column (7.5 $\times$ 600 mm, Pharmacia, Sweden) equilibrated with TENG buffer. Chromatography was performed in a high-performance liquid chromatography (HPLC9) (Pharmacia, Sweden) at 0.2 mL/min flow.

Electron microscopy. Purified HCcAg was fixed in glutaraldehyde and negatively stained with uranyl acetate prior to analysis by transmission electron microscopy as described before (19).

Immunization schedule. Two sheep were immunized with 3 doses of 200 μg of HCcAg in incomplete Freund's adjuvant. The doses were applied at weeks 0, 2, and 5. Sera were obtained from immunized sheep to study anti-HCcAg antibody response at weeks 0, 2, 5, and 8.

Assay for determination of anti-HCV core antibody response. An indirect ELISA to measure anti-HCcAg antibodies in sheep's serum was developed. In brief, HCV-core protein (10 μg/ml) diluted in 50 mM carbonate buffer (pH 9.6) was used to coat microtiter plates (Costar, U.S.A.), overnight at 4°C. After blocking with 2% (w/v) skim milk powder (Oxoid Ltd., England) in PBS with 0.05% (v/v) Tween 20 (Sigma) (PBST), pH 7.2 for 1 h at room temperature. The serum samples were added in duplicate, either 1/20 in dilution buffer (PBST), containing 1% (w/v) skim milk powder, to test seroconversion or in a double serial starting at 1/50 dilution for titration. They were incubated at 37°C for 1 h. A horseradish peroxidase (HRP)-labeled anti-sheep IgG (Sigma) was added to 1/10,000 in the dilution buffer. After 1 h of incubation at room temperature (RT), *o*-phenylenediamine (OPD) (Sigma) substrate diluted in phosphate–citrate buffered solution (pH 5.6) was added for color development, and incubated at RT for 30 min. All washing steps were carried out three times with PBST.

Reactions were stopped with 50 μl of 2.5 M H_2SO_4. Absorbance was read at 492 nm in a SensIdent Scan (Merck, Germany). The cut-off value used to consider a positive anti-core antibody response was established as twice the mean of absorbance values of the preimmune sera.

RESULTS AND DISCUSSION

The exact mechanisms behind the ability of HCcAg to assemble into a nucleocapsid structure are still unknown. One hypothesis is that HCcAg has the intrinsic quality for self-assemble into VLPs. Viral core proteins usually multimerize to form a nucleocapsid, thus providing a protective shell for the viral genome inside. HCcAg is expected to have similar properties. Previous studies had indicated that HCcAg can directly interact with itself by specific homotypic interaction, and forms multimers both *in vitro* and *in vivo* (9), as well as nucleocapsids *in vitro* (10). Recombinant HCV core proteins expressed in *Escherichia coli* (25), and *P. pastoris* cells (19, 20) spontaneously associate to form capsids in the absence of additional HCV proteins. Our study was carry out to prove that HCcAg could reassemble *in vitro* into VLPs by itself.

To express HCcAg (Fig. 1), the MP-36/C-E1.339 strain (19) was grown as described above. Prior we had demonstrated that the HCcAg expressed in *P. pastoris*

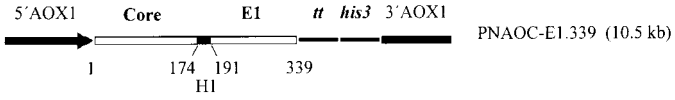

FIG. 1. Schematic representation of the expression vector pNAO.COE1.339 used for transforming the *P. pastoris* strain MP-36. 5′AOX1 for the expression of HCcAg. 5′AOX1, methanol oxidase promoter; *tt*, transcription terminator; AA, amino acid; core and E1, HCV structural core and envelope 1, respectively.

cells was mainly found associated to membrane components (19). Cells were disrupted in denaturing conditions, to ensure the extraction and depolymerization of HCcAg. To purify HCcAg as a low molecular weight species and avoid interaction with any nucleotides, HCcAg was kept under denaturing conditions and heated for 30 min. Then it was purified by electroelution using SDS–PAGE (Bio-Rad) in denaturing conditions (Fig. 2A). This protein was specifically recognized by mAb SS-HepC.1 (Fig. 2B). After purification both denaturalized and renaturalized HCcAg were analyzed by electron microscopy. The presence of spherical particles with uniform morphology and size distribution with an average diameter of 30 nm were observed for the renaturalized HCcAg (Fig. 3A). However, these VLPs were not observed in the fully denaturalized HCcAg (Fig. 3B). The average diameter observed for VLPs containing HCcAg is similar with those found in infected patients (26), thus resembling HCV nucleocapsid particles in a mature stage.

To determine the efficiency and homogeneity of the self-assembled structure, we performed size-exclusion chromatography. As a control, the HBsAg (24) was also analyzed under the same conditions. The HCcAg was detected as a uniform peak close to the fraction corresponding to the molecular exclusion size of HBsAg (Fig. 4A). This demonstrated the homogeneity of self-assembled particles. Additionally, the fraction containing HCcAg was specifically recognized by antibodies from a chronically HCV infected patient (Fig. 4B).

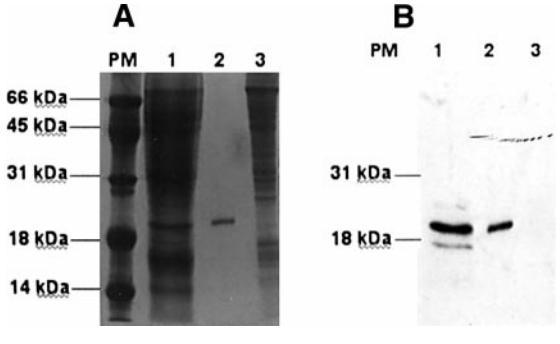

FIG. 2. Results of the HCcAg purification by electroelution. (A) Proteins were separated by SDS–PAGE. (B) Western blot of HCcAg using a mouse monoclonal antibody mAb SS-HepC.1. The lanes contain size markers (PM), the protein sample containing HCcAg extracted with urea from cell debris (1), the purified HCcAg (2), and the protein sample extracted with urea from MP-36 strain cell debris, used as a negative control.

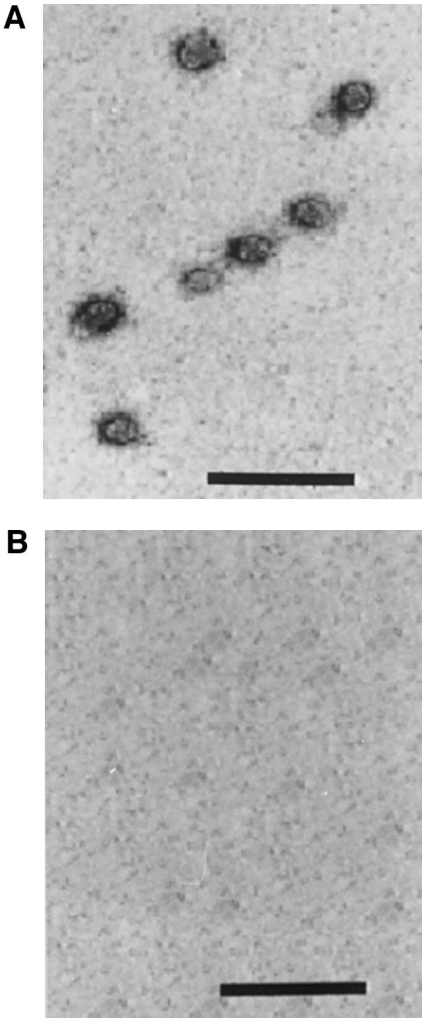

FIG. 3. Electron micrograph of negatively stained VLPs containing HCcAg after renaturalization (A) and denatured HCcAg (B). Bar, 100 nm.

The fact that HCV core-like particles can be assembled *in vitro* indicates that the core protein itself contains all information necessary for the self-assembly process. A previous study reported that two variants of purified HCcAg comprising 124 and 179 aa, respectively, obtained from *E. coli* cells, assembled *in vitro* into VLPs (10). It was found that self-assembly of VLPs required structured RNA molecules. In our system, assembly of HCcAg into VLPs did not require the addition of nucleic acids. Renaturalized HCcAg was analyzed in agarose gel, and not ethidium bromide-stained bands were seen (data not shown). Although we could not completely discard that limited amounts of undetected contaminant nucleic acids influences the self-assembly properties of HCcAg in our system, it seems that the conditions in which HCcAg was purified avoided the inclusion of nucleotides inside VLPs. The

use in our work of an intracellular processed HCcAg (19), similar to the processed p21 HCcAg involved in *in vivo* nucleocapsid assembly, may explain the differences with previous work (10). Thus, we suggest that, at least *in vitro,* assembly of HCV nucleocapsids not necessarily have to occur simultaneously with encapsidation of viral RNA. For other viral systems it has been suggested that self-assembly of core proteins could precedes the interactions between the viral HCcAg and the viral RNA (27). This model predicts the existence of preformed empty capsids proteins as intermediates in encapsidation. Though for HCV a model like this remains to be further demonstrated, we propose that a similar mechanism may occur for HCV nucleocapsid assembly, alternatively or in addition to the previous finding which demonstrated that *in vitro* assembly of HCV nucleocapsids and encapsidation of RNA occurs simultaneously (10). Little is known about the mechanism of HCV assembly, virus structure and the virion composition. Thus, the approach above described would be helpful to elucidate some of the mechanisms underlying these topics.

On the other hand, particulate or aggregate antigens have proved to be efficient in generating powerful and long-lasting immune responses (28). The HCcAg could be of interest as a vaccine since it is the most conserved HCV protein among genotypes and is highly immunogenic (29). Two sheep were immunized with HCcAg to study immunogenicity. While sera from pre-immune samples did not show any reactivity to HCcAg, the anti-HCV core IgG response rose rapidly from week 2 to 8, and titers above 1/100,000 were reached in both immunized animals (Fig. 5). This result demonstrates that these *in vitro* self-assembled VLPs are capable of inducing a strong antibody immune response. However, further immunological studies will be necessary to prove this antigen as a useful vaccinal candidate.

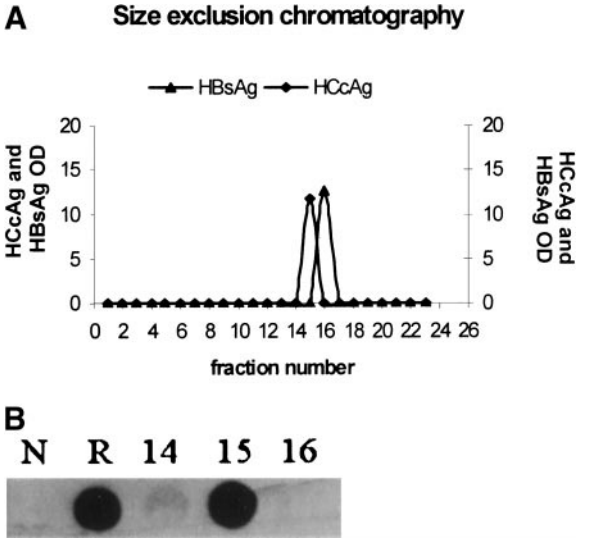

FIG. 4. Size-exclusion chromatography on TSK G5000 column. (A) Chromatography lots of the purified HCcAg after renaturalization and HBsAg. Values for HCcAg and HBsAg are in micrograms. (B) Dot blot of the HCcAg using a human serum from a chronic patient. N contains the HBsAg used as a negative control, and R contains the renaturalized HCcAg loaded onto the TSK G5000 column. 14, 15, and 16 contain chromatography fractions from 14 to 16.

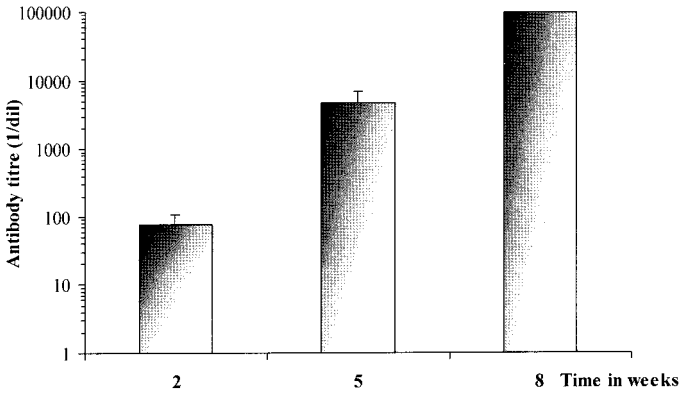

FIG. 5. The time course of the anti-HCcAg IgG response after immunization is shown. Results are shown as the reciprocal of the serum dilution equivalent to twice the mean optical density ($OD_{492\ nm}$) of negative control. For clarity, errors bars indicating the standard errors of the means are shown in the positive sense only.

It was concluded that the *in vitro* assembly of VLPs containing HCcAg that resemble HCV nucleocapsid particles in a mature stage is an intrinsic quality of this protein. Immunization with HCcAg generates a strong antibody immune response in sheep, suggesting its usefulness in stimulating a host immune response against HCV.

ACKNOWLEDGMENTS

The authors thanks Professor Orlando J. Alvarez Guerrero, Dr. Alejandro Moro, and Dr. Julio C. Aguilar for critical reading of the manuscript and for many helpful suggestions.

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