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# Chimeric Hepatitis B Virus / Hepatitis C Virus Envelope Proteins Elicit Broadly Neutralizing Antibodies and Constitute a Potential Bivalent Prophylactic Vaccine

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*Abbreviations: CHO, chinese hamster ovary cell line; FFU, focus-forming unit; HBV, hepatitis B virus; HCV, hepatitis C virus; HCVcc, hepatitis C virus in cell culture; HCVpp, hepatitis C virus pseudoparticles; IFN, interferon; Ig, immunoglobulin; mAb, monoclonal antibody; NAbs, neutralizing antibodies; OD, optical density; HBsAg, hepatitis B surface antigen; SFV, semliki forest virus; TMD, transmembrane domain.*

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# Abstract

**The development of a prophylactic vaccine against hepatitis C virus (HCV) has become an important medical priority, as three to four million new HCV infections are thought to occur each year worldwide. The hepatitis B virus (HBV) is another major human pathogen, but infections with this virus can be prevented with a safe, efficient vaccine, based on the remarkable ability of the envelope protein (S) of this virus to self-assemble into highly immunogenic subviral particles. Chimeric HBV-HCV envelope proteins in which the N-terminal transmembrane domain of S was replaced with the transmembrane domain of the HCV envelope proteins (E1 or E2) were efficiently coassembled with the wild-type HBV S protein into subviral particles. These chimeric particles presented the full-length E1 and E2 proteins from a genotype 1a virus in an appropriate conformation for formation of the E1-E2 heterodimer. Produced in stably transduced CHO cells and used to immunize New Zealand rabbits, these particles induced a strong specific antibody response against the HCV and HBV envelope proteins in immunized animals. Sera containing anti-E1 or anti-E2 antibodies elicited by these particles neutralized infections with HCV pseudoparticles and cell-cultured viruses derived from different heterologous 1a, 1b, 2a and 3 strains. Moreover, the anti-HBs response induced by these chimeric particles was equivalent to the response induced by a commercial HBV vaccine.** ***Conclusions:*** **Our results provide support for approaches based on the development of bivalent HBV-HCV prophylactic vaccine candidates potentially able to prevent initial infection with either of these two hepatotropic viruses.**

Chronic hepatitis C virus (HCV) infection is a major public health problem affecting more than 170 million people worldwide (1). Three to four million new infections are thought to occur each year and a prevalence of 10 to 30% has been reported in countries in which this virus is highly endemic, including Egypt, which has the highest HCV prevalence in the world (2). HCV infection is one of the leading causes of chronic liver disease; it is associated with a high risk of cirrhosis and hepatocellular carcinoma and is the major indication for liver transplantation in industrialized countries. Provided it is detected sufficiently early, progression to severe disease can be prevented by treatment with a combination of pegylated interferon (IFN)-α and ribavirin, in some cases supplemented with recently approved NS3/4A protease inhibitors (3). This triple therapy yields a sustained virologic response but is very expensive and may be associated with drug-drug interactions and severe side effects. The development of a prophylactic vaccine against HCV is therefore a major medical priority. However, the development of such a vaccine is very challenging. The HCV RNA genome is highly diverse, both within and between isolates, and three levels of classification have been adopted, grouping viral sequences into six genotypes, a few dozen subtypes and intra-isolate variants (4). However, several other features may facilitate vaccine development. The virus is spontaneously eradicated by about 15 to 25% of acutely infected individuals (5) and significant levels of natural immunity to HCV have been reported in studies of the chimpanzee model of HCV infection (6) and in studies of reinfections in intravenous drug users (7). The resolution of HCV infection is frequently associated with robust cellular immunity (8), but many studies have also highlighted an important role for neutralizing antibodies (NAbs) (9).

The HCV envelope glycoproteins, E1 and E2, exposed on the surface of viral particles, are involved in virus-host interactions and constitute potential targets for NAbs. Houghton and coworkers showed that a prophylactic vaccine strategy based on the use of recombinant E1 and E2 with adjuvant effectively protected immunized chimpanzees against experimental

intravenous challenge and/or HCV-associated disease (10,11). These vaccinated chimpanzees produced high titers of cross-neutralizing antibodies targeting epitopes broadly conserved within the diverse *Hepacivirus* genus (12). This recombinant E1-E2 vaccine was well tolerated and immunogenic in human volunteers (13). However, these immunogens have a transmembrane domain and are retained in intracellular compartments in the form of a large, non covalently linked heterodimer, rendering their extraction and purification extremely difficult and incompatible with industrial development for vaccination purposes (14). These difficulties have led to alternative strategies based on the use of truncated E1 and/or E2 proteins. The production of the ectodomain of E1 and E2 alone is sufficient to ensure protein secretion, but deletion of the transmembrane domains of these proteins has been shown to impair their antigenic and functional properties (15). Another type of vaccine platform based on retroviral Gag particles pseudotyped for HCV envelope proteins has recently been proposed (16). These retroviral particles induce high titers of anti-E2 and/or anti-E1 antibodies in mice and macaques, and NAbs raised against HCV 1a cross-neutralize five other genotypes *in vitro*. However, the use of animal retroviral particles for the development of a prophylactic vaccine for humans will require validation, and it may prove difficult to mass produce such a vaccine.

The small (S) envelope protein of hepatitis B virus (HBV), HBsAg, self-assembles into highly immunogenic, noninfectious and secreted subviral particles, which have been used worldwide as a safe, commercial hepatitis B vaccine since the early 1980s (17). In many countries with pre-vaccination rates of chronic HBV infection in children of up to 15%, vaccination has reduced these rates to less than 1% in immunized children. In 2009, the WHO reported that 177 countries had included the HBV vaccine in their national infant immunization programs (http://www.who.int/immunization/topics/hepatitis_b/en/). In an original strategy for incorporating the full-length HCV E1 and E2 proteins into virus-like particles, we recently described the possible replacement of the N-terminal transmembrane domain (TMD) of the

HBV S protein with the transmembrane domain of E1 or E2, to form a chimeric protein able to self-assemble with the wild-type S protein to produce a secreted particle (18). We show here that the resulting HBV-HCV subviral particles, presenting the genotype 1a E1 and E2 proteins, elicit high titers of antibodies capable of neutralizing various HCV genotypes. These particles also induced high titers of anti-HBs antibodies, providing further support for the development of a bivalent HBV-HCV vaccine.

## Materials and Methods

***Production of chimeric HBV-HCV subviral envelope particles and immunogens.*** Chimeric HBV (adw genotype) – HCV (1a genotype) envelope particles bearing the E1-S or E2-S proteins, produced from two different Chinese hamster ovary (CHO) clones (Fig. 1A) have been described elsewhere (18). For the selection of a CHO clone constitutively producing the wild-type HBV S and both chimeric E1-S and E2-S proteins, the CHO-S+E1-S clone was stably transduced with a lentiviral expression vector encoding the chimeric E2-S protein and the puromycin N-acetyltransferase. A cell clone resistant to both puromycin and hygromycin, CHO-S+E1-S+E2-S (Fig. 1B), was amplified and analyzed as previously described (18).

Basic protocols for HBV subviral envelope particle purification were used for immunogen preparation. Briefly, secreted particles were purified from the supernatants by centrifugation on CsCl gradients. HBsAg-positive fractions were pooled, dialyzed at 4°C against Tris-NaCl-EDTA (TNE; 10 mM Tris/HCl pH 7.5/ 100 mM NaCl/ 1 mM EDTA) and concentrated with an Amicon® Ultracell-100k (Millipore) device. Protein concentration in these final preparations was determined in a colorimetric DC protein assay (Bio-Rad), and HBsAg levels were determined in a quantitative microparticle chemiluminescence immunoassay (ARCHITECT System; Abbott). The presence of HBV S and HBV-HCV chimeric E1-S and E2-S proteins in the purified particles was investigated by western blotting, as previously described (18).

***Analysis of HCV envelope proteins folding and heterodimerization in the context of the fusion proteins.*** We developed an ELISA using human monoclonal antibodies (mAbs) AR3A and AR5A, which recognize conformation-dependent discontinuous epitopes on the E2 protein and the folded E1-E2 complex, respectively (19,20). AR3A and AR5A (2 µg/ml) were added to ELISA wells coated with various chimeric particles (25 µg/ml) or JFH-1 WT viruses purified on sucrose cushions (1,000 FFU) captured with pre-coated lectin from Galanthus Nivalis (20 µg/ml). Specific binding was detected with a peroxidase-conjugated mouse anti-human immunoglobulin (Ig) mAb (clone JDC-10) diluted 1:2,000.

***Immunization of New Zealand rabbits.*** Fifteen micrograms of immunogens (S+E1-S, S+E2-S or S+E1-S+E2-S), quantified by HBsAg ELISA, were mixed immediately before immunization with a squalene-based oil-in-water nanoemulsion (Addavax®; Cayla-Invivogen). This adjuvant has a formulation similar to that of the MF59 adjuvant which is approved for human use, and has been shown to give a stronger humoral immune response than aluminum salts (21). Groups of six rabbits were immunized subcutaneously with the immunogen-adjuvant mixture on days 0, 14 and 28. Groups of three rabbits immunized with the Addavax adjuvant alone or with Engerix®, a commercially available HBV vaccine, were used as negative and positive controls, respectively. Serum samples were collected from the rabbits at various time points (days 0, 14, 28, 42 and 56), to characterize the antibody responses. All animal experiments were conducted by an accredited company (Agro-bio, France), in accordance with current guidelines for animal experimentation.

***Analysis of anti-S and anti-E1E2 antibody responses.*** Anti-HBs antibodies were quantified with the ARCHITECT system (Abbott). Anti-E1E2 responses were evaluated with an "in-house" ELISA described in the supporting materials and methods.

***Neutralization assays with HCVcc and HCVpp.*** HCVcc harboring HCV envelope glycoproteins derived from genotype 1a (JFH1/H77), 1b (JFH1/J4), 2a (JFH1 WT) and 3

(JFH1/S52) isolates (22,23) were used to assess and compare the neutralizing activity of pre- and post-vaccination rabbit sera. Neutralization data obtained with the HCVcc model were compared with those obtained with the HCVpp model, using HCVpp harboring HCV envelope glycoproteins derived from genotype 1a (7a), (24) 1b (UKN5.23), 2a (UKN 2a1.2 ) and 3 (UKN3A.1.28) (25) isolates. For some experiments, we also used purified antibodies from selected rabbit sera. All these assays are described in the supporting materials and methods.

***CD81-binding inhibition analysis.*** *In vitro* binding assays were conducted to characterize the CD81-binding properties of the chimeric S+E2-S or S+E1-S+E2-S particles and to assess the ability of NAbs in the rabbit sera to inhibit this interaction. These assays are described in the supporting materials and methods.

## Results

***Production of chimeric HBV-HCV envelope particles and analysis of the final immunogen preparations.*** The transduced CHO clones stably produced more than 10 µg/ml HBsAg over several months. Following particle purification, the proteins of interest (HBsAg and chimeric HBV-HCV proteins) accounted for 10% of total protein, on average. Electron microscopy (EM) analysis of these preparations showed that the secreted particles were similar in size (about 22 nm in diameter) and appearance to those present in a commercially available HBV vaccine (Fig. 1A). Finally, western blotting with a polyclonal antibody against HBsAg (R247) or mouse mAbs against E1 (A4) or E2 (H52) confirmed that the chimeric E1-S (p54) and/or E2-S (p85) proteins were efficiently incorporated into the particles produced by these CHO clones in long-term culture (Fig. 1A and 1B).

***Efficient folding and heterodimerization of the HCV envelope proteins in the context of the fusion proteins.*** S+E2-S and S+E1-S+E2-S chimeric particles were specifically and efficiently recognized by a neutralizing, conformation-dependent anti-E2 mAb (AR3A), with

an efficacy similar to that for the JFH1 WT virus (Fig.2). Moreover, the signal for S+E1-S+E2-S particles was significantly stronger than that for S+E2-S particles. By contrast, no binding was observed with the S and S+E1-S chimeric particles, attesting to the specificity of the observed signals (Fig. 2). The neutralizing mAb AR5A, previously shown to recognize the E1-E2 complex (20), specifically interacted with our S+E1-S+E2-S particles (Fig. 2), attesting to the presence of the E1-E2 heterodimer at the surface of these particles, despite the fusion of the E1 and E2 proteins with the HBV S protein.

***Induction of a humoral immune response in rabbits immunized with AddaVax®-adjuvanted chimeric S+E1-S, S+E2-S or S+E1-S+E2-S envelope particles.*** Antibodies directed against E1 and E2 were detected in the sera of almost all (17 of 18) the rabbits immunized three times with the S+E1-S, S+E2-S or S+E1-S+E2-S particles, although the responses in S+E2-S-immunized rabbits were more sustained than those in rabbits immunized with S+E1-S or S+E1-S+E2-S (Fig. 3A). This suggested that the particles containing the S+E1-S protein were less immunogenic than those containing only the S+E2-S protein. The kinetics of antibody production varied between animals. All rabbits presenting a humoral anti-E1 or anti-E2 response also displayed a strong humoral anti-HBsAg response, equivalent to that observed in rabbits immunized with a commercial HBV vaccine (Fig. 3B). The presence of the E1-S protein seemed to decrease HBsAg immunogenicity in some rabbits immunized with S+E1-S or S+E1-S+E2-S particles.

***Cross-neutralizing properties of antibodies induced by immunization with AddaVax®-adjuvanted chimeric S+E1-S, S+E2-S or S+E1-S+E2-S particles.*** All the rabbit sera containing anti-E1 and/or anti-E2 antibodies had cross-neutralizing properties *in vitro* against HCVcc harboring heterologous HCV envelope proteins derived from strains of different genotypes. These antibodies neutralized heterologous genotype 1a and 1b antigens more efficiently than those from viruses of other genotypes, but significant cross-neutralization was

nonetheless observed for the heterologous genotypes 2a and 3 (Fig. 4). The neutralizing activities of antibodies produced in response to immunization with S+E1-S+E2-S particles were similar to those generated by immunization with S+E2-S particles, but much stronger than those induced by immunization with S+E1-S particles. By contrast, sera from rabbits immunized with Engerix® and adjuvant alone had no effect on the infectivity of HCVcc (Fig. 4). We also demonstrated with additional neutralizing assays using five-fold serial dilutions of rabbit sera collected on days 0 and 56 that a significant specific neutralizing activity against HCVcc could still be observed for high serum dilutions (Fig.5). All these results were confirmed with the HCVpp model (Supporting Fig. S1 and supporting Fig.S2), although HCVcc was two to three times more sensitive to neutralization than HCVpp derived from genotypes 1a, 1b, 2a and 3. Moreover, to exclude a possible role of other factors present in the rabbit sera, we also performed neutralization experiments with purified antibodies. Similar results were obtained for both the HCVcc (Fig. 6) and HCVpp (Supporting Fig. S3) models, demonstrating unambiguously that the neutralizing signal was due to NAbs in the rabbit sera.

***CD81-binding inhibition properties of antibodies induced by immunization with chimeric particles.*** In a first set of experiments investigating the capacity of S+E2-S and S+E1-S+E2-S chimeric particles to bind CD81, both particles bound this cellular receptor specifically. As the binding signal for S+E1-S+E2-S particles was slightly stronger than that for S+E2-S particles, subsequent CD81-binding inhibition assays were conducted with S+E1-S+E2-S particles. Only the anti-E2 antibodies in the rabbit sera and the mAb AR3A, previously reported to recognize conformational epitopes overlapping the CD81 binding site (19), were shown to inhibit the binding of the S+E1-S+E2-S particles and the JFH1 WT virus to CD81 partially, with different efficiencies (Fig. 6). By contrast, anti-E1 antibodies present in rabbit sera and the mAb AR5A, previously reported to recognize conformational epitopes not overlapping the CD81 binding site (20), did not interfere with CD81 binding (Fig. 6).

## Discussion

With three to four million new HCV infections occurring annually worldwide, including 18,000 in the USA (26), the development of a prophylactic vaccine against HCV is an important medical priority. Many studies have highlighted the importance of both cellular and neutralizing responses in HCV clearance (8,9), but the induction of broadly neutralizing antibodies remains the ultimate goal for prophylactic vaccines against HCV (27). NAbs mostly recognize tertiary or quaternary structures, so any vaccination strategy designed to induce NAbs would require the target proteins to be produced in the correct conformation. We therefore developed a strategy for incorporating full-length HCV E1 and E2 proteins into HBV subviral envelope particles (18). The insertion of a small peptide into the antigenic external loop of the HBV S protein or the fusion of a protein at the N or C-terminus of this protein has been reported in several original vaccine strategies (28,29). However, in our approach, we designed chimeric HBV-HCV envelope proteins in which the N-terminal TMD of the HBV S protein was replaced with the TMD of E1 or E2. These chimeric HBV-HCV envelope proteins, containing full-length E1 and E2 proteins, were efficiently incorporated into subviral particles when produced together with the wild-type HBV S protein. Like the wild-type HBV S subviral particles used in current HBV vaccines, these chimeric particles were efficiently secreted into the cell supernatant and were easily purified. We designed plasmids encoding chimeric E1-S or E2-S envelope proteins individually, to investigate the contribution of each of the entire HCV E1 and E2 proteins to the humoral response, and to neutralization in particular. We adopted this approach because E2 and E1 are clearly targets of NAbs (30,31), and little is known about anti-E1 antibody responses in HCV-infected patients. E1 is a particularly interesting target for NAb induction because it displays little variability and the degree of inter-genotype cross-reactivity may be higher for this protein than for E2 (32). Furthermore, the detection of anti-E1

monoclonal cross-neutralizing antibodies has been reported (33). However, it seems to be very difficult to trigger a strong anti-E1 response (16), consistent with the observation that high levels of anti-E1 antibodies are rarely detected in patients (9).

We first characterized, through *in vitro* assays, the folding and potential heterodimerization of the full-length HCV envelope proteins presented at the surface of the chimeric particles. Using the broadly neutralizing mAbs AR3A and AR5A previously reported to recognize conformation-dependent discontinuous epitopes on E2 and the folded E1-E2 complex, respectively (19,20), we clearly demonstrated that the HCV envelope proteins were productively folded in the HBV S fusion proteins, generating E1-E2 heterodimers at the surface of S+E1-S+E2-S particles. Moreover, the stronger AR3A-binding signal observed with S+E1-S+E2-S particles than with S+E2-S particles confirmed the involvement of E1 in critical steps for productive E2 folding (34).

The main objective of this study was to evaluate, through *in vitro* assays, the immunogenicity of these vaccine candidates and their ability to induce neutralizing antibodies against HCV in a small-animal model. Our findings clearly demonstrate that S+E1-S and S+E2-S chimeric envelope subviral particles induce a strong immune response against HBV and HCV envelope proteins in rabbits. The response was less sustained in S+E1-S-immunized rabbits than in S+E2-S-immunized rabbits, suggesting that E2 is more immunogenic than E1. These data are consistent with previous studies indicating that E1 is only weakly immunogenic or induces non neutralizing antibodies (16,35). Furthermore, the mere presence of E1 was sufficient to impair HBsAg immunogenicity.

Using the HCVpp (24) and HCVcc (22) [2a JFH-1 strain and its derivatives, through the use of chimeric genomes encoding structural proteins from different genotypes (23)] models, we demonstrated that our chimeric S+E1-S and S+E2-S particles, containing the envelope of genotype 1a HCV, elicited high titers of antibodies that neutralized HCVcc and HCVpp

harboring E1 and E2 from different heterologous genotype 1a, 1b, 2a and 3 strains. These antibodies appeared to neutralize the 1a and 1b genotypes efficiently, and the 2a and 3 genotypes less efficiently. These data highlight the major differences separating genotypes 2a and 3 from genotype 1, accounting for the potentially high heterogeneity of the epitope sequences recognized, as previously described (12). However, our data suggest that these cross-neutralizing antibodies target several epitopes broadly conserved in diverse HCV strains. The response induced by immunization with S+E1-S particles was much weaker than that induced by S+E2-S particles, but E1 induced a strong immune response when used alone, including the production of antibodies with neutralizing properties. Our data are consistent with those from a recent study showing that anti-E1 antibodies induced by immunization with a recombinant form of E1 confer protection against experimental infection with heterologous HCV strains in chimpanzees (36). However, the authors of this previous study reported that the E1-based vaccine conferred stronger protection than the E2-based vaccine. In future experiments, it will be important to identify the epitopes targeted by the cross-neutralizing antibodies induced by various vaccine candidates and the stages in the viral lifecycle affected by these antibodies.

Immunization results suggested that both E1 and E2 elicited strong immune responses, but we observed no synergistic effects or cooperation between these proteins when presented together at the surface of S+E1-S+E2-S particles. This observation is consistent with data from previous studies indicating that an anti-E1 response is difficult to trigger and that separate E1 and E2 immunizations are required to elicit an anti-E1 antibody response (16,35). It has been suggested that E2 is immunodominant or that E1 immunogenic epitopes are masked in the presence of E2. The humoral, neutralizing response induced by immunization with S+E1-S+E2-S particles in this study was similar to that observed after immunization with S+E2-S particles, demonstrating that both S+E2-S and S+E1-S+E2-S particles are good immunogens for our HBV-HCV vaccine strategy.

We explored the HCV-neutralizing mechanisms of the anti-E2 antibodies in our rabbit sera. These NAbs inhibited E2/CD81 binding only partially, suggesting action at both pre- and post-binding entry steps. These data are consistent with studies indicating that neutralizing responses in HCV-infected patients target post-binding entry steps (37), and preliminary neutralization assays with our rabbit sera in experimental conditions distinguishing between antibody-mediated interference during binding and post-binding events (data not shown).

Our data confirm the existence, reported elsewhere (16), of a large difference in neutralization sensitivity between the HCVpp and HCVcc models. There are several possible reasons for this difference. HCV envelope proteins may differ in conformation and glycosylation pattern, even if derived from the same HCV strain. E1 and E2 form non covalent heterodimers at the surface of HCVpp (38), and these heterodimers were thought to be the mature functional forms on the virus surface. However, more recent studies investigating E1 and E2 in HCVcc particles, which are thought to be more similar to the native HCV virion, indicated that larger covalent complexes stabilized by disulfide bridges were present (39). It remains to be determined whether the higher efficiency of HCVcc neutralization by the NAbs induced by our HBV-HCV chimeric particles results from the presence of these large covalent complexes at the surface of the HCVcc particles. Nevertheless, despite these differences in neutralization intensities, the NAbs induced by our chimeric HBV-HCV particles clearly cross-neutralized different HCV genotypes in both these models.

In conclusion, the encouraging data generated by this study support the development of a bivalent HBV-HCV prophylactic vaccine candidate. Further experiments are required to investigate, in different animal models, the specific HCV-neutralizing response induced by these particles, comparing this response with that to purified recombinant E1-E2 heterodimers (10,11,40). However, our bivalent vaccine candidate would be of considerable interest, because the populations at risk of infection with HBV and HCV through exposure to infected blood are

essentially the same: intravenous drug users, healthcare providers, medical technicians, military personnel and policemen (41). In this context, the induction by HBV-HCV envelope particles of an anti-HBs response equivalent to that induced by commercially available HBV vaccines is also important, as it suggests that bivalent HBV-HCV vaccines could replace existing vaccines against HBV whilst providing the additional benefit of protection against HCV. Another major advantage of this approach is that this vaccine candidate could be produced by the same procedures established for HBV vaccines, reducing the time and cost of its industrial development. Studies of the immunization of rabbits and other small-animal models with a pool of chimeric HBV-HCV particles bearing HCV envelopes from different genotypes are currently underway, with the objective of increasing the broadly neutralizing properties of the NAbs induced by this vaccination strategy.

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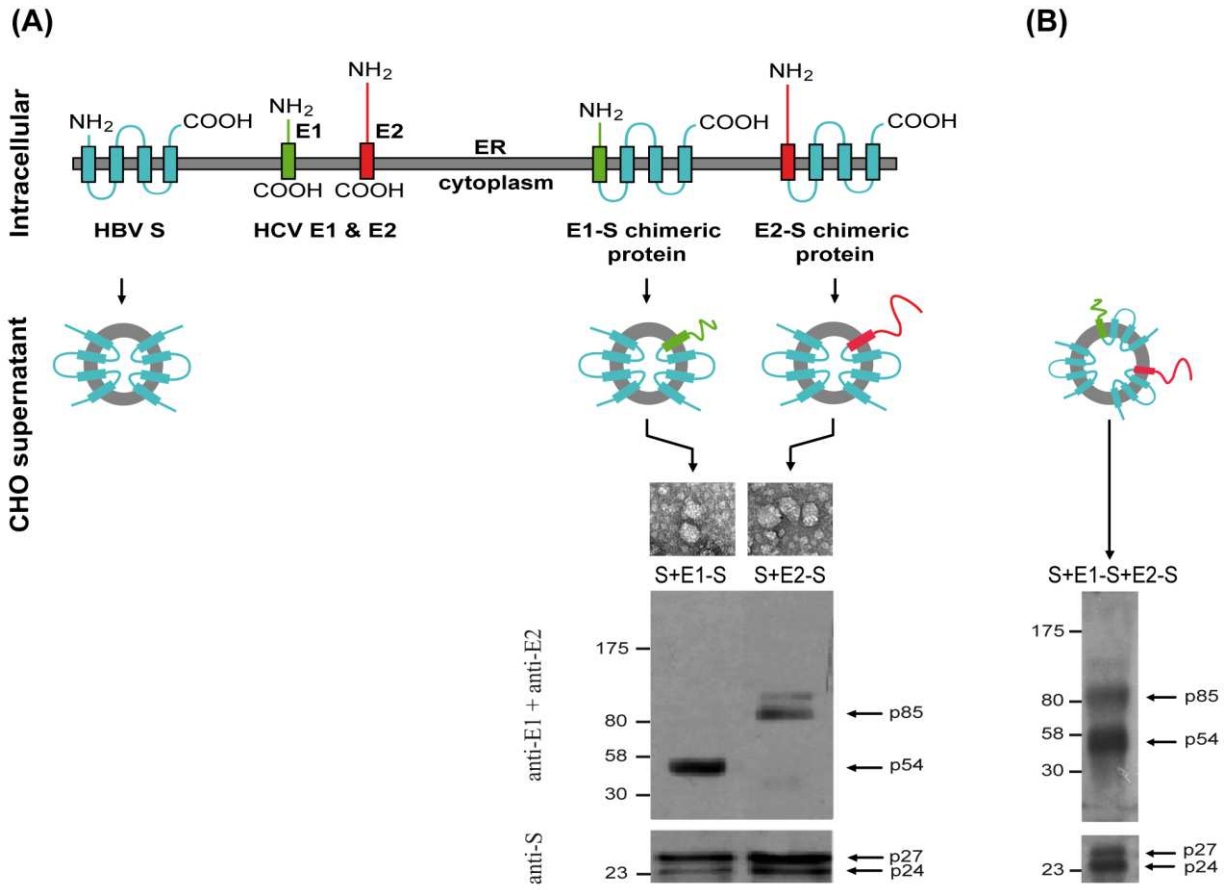



**Fig. 1.** Chimeric HBV-HCV envelope particles used as immunogens. Chimeric HBV-HCV envelope proteins, in which the N-terminal TMD of the HBV S protein was replaced with the TMD of E1 or E2 self-assembled with the wild-type HBV S protein to form a secreted particle. Western-blot analysis of purified particles demonstrated the efficient incorporation of chimeric HBV-HCV envelope proteins (E1-S or/and E2-S) into the secreted S+E1-S, S+E2-S **(A)** and S+E1-S+E2-S **(B)** subviral particles.

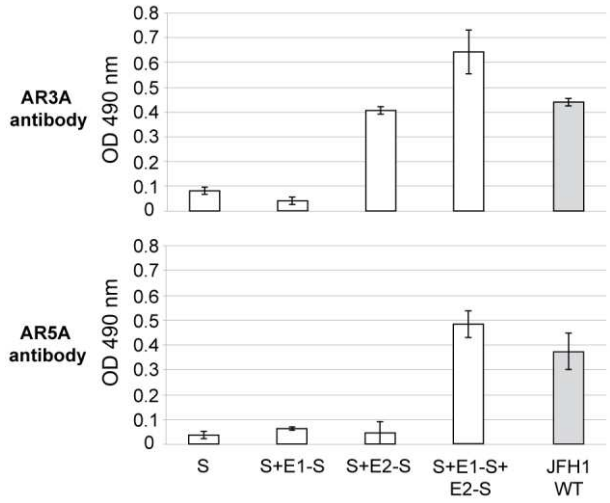



**Fig. 2.** Productive folding and heterodimerization of the HCV envelope proteins in the context of the fusion proteins. The mAbs AR3A and AR5A were added to ELISA wells coated with chimeric particles (S, S+E1-S, S+E2-S or S+E1-S+E2-S; 25 µg/ml) or JFH-1 WT viruses purified on sucrose cushions (1,000 FFU; positive control: gray histogram) captured with a lectin precoating. Specific binding was detected with monoclonal peroxidase-conjugated mouse anti-human immunoglobulin antibody. Absorbance at 490 nm was determined. The data shown are the means ± SD of two independent experiments performed in triplicate.

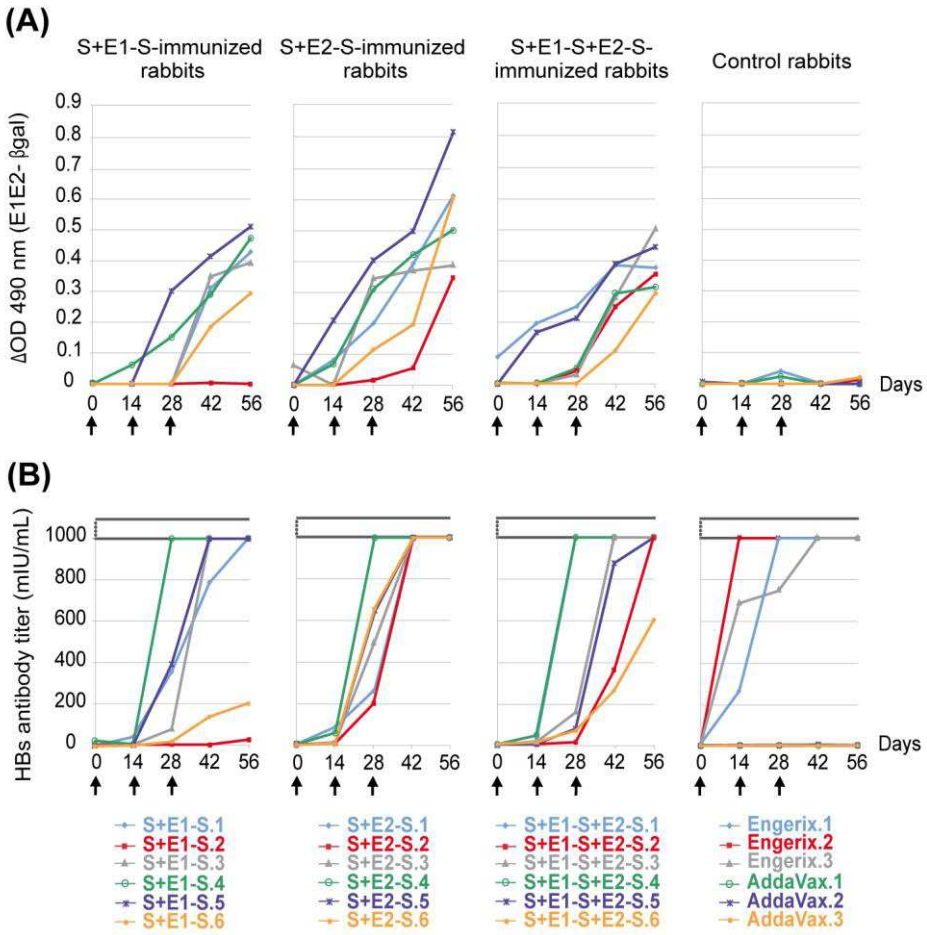



**Fig. 3.** Humoral immune responses induced in rabbits immunized with chimeric S+E1-S, S+E2-S or S+E1-S+E2-S envelope particles in the presence of AddaVax®. **(A)** Anti-E1/E2 and **(B)** anti-HBs responses were evaluated on rabbit sera collected at various time points with an "in-house" ELISA and a routine immunoassay (Abbott), respectively. Sera from rabbits immunized with the adjuvant alone or with Engerix® were used as negative and positive controls, respectively. Black arrows indicate the time at which immunization occurred. Results are expressed as the difference in OD (E1E1-β-gal) and anti-HBs titer (mIU/ml), respectively.

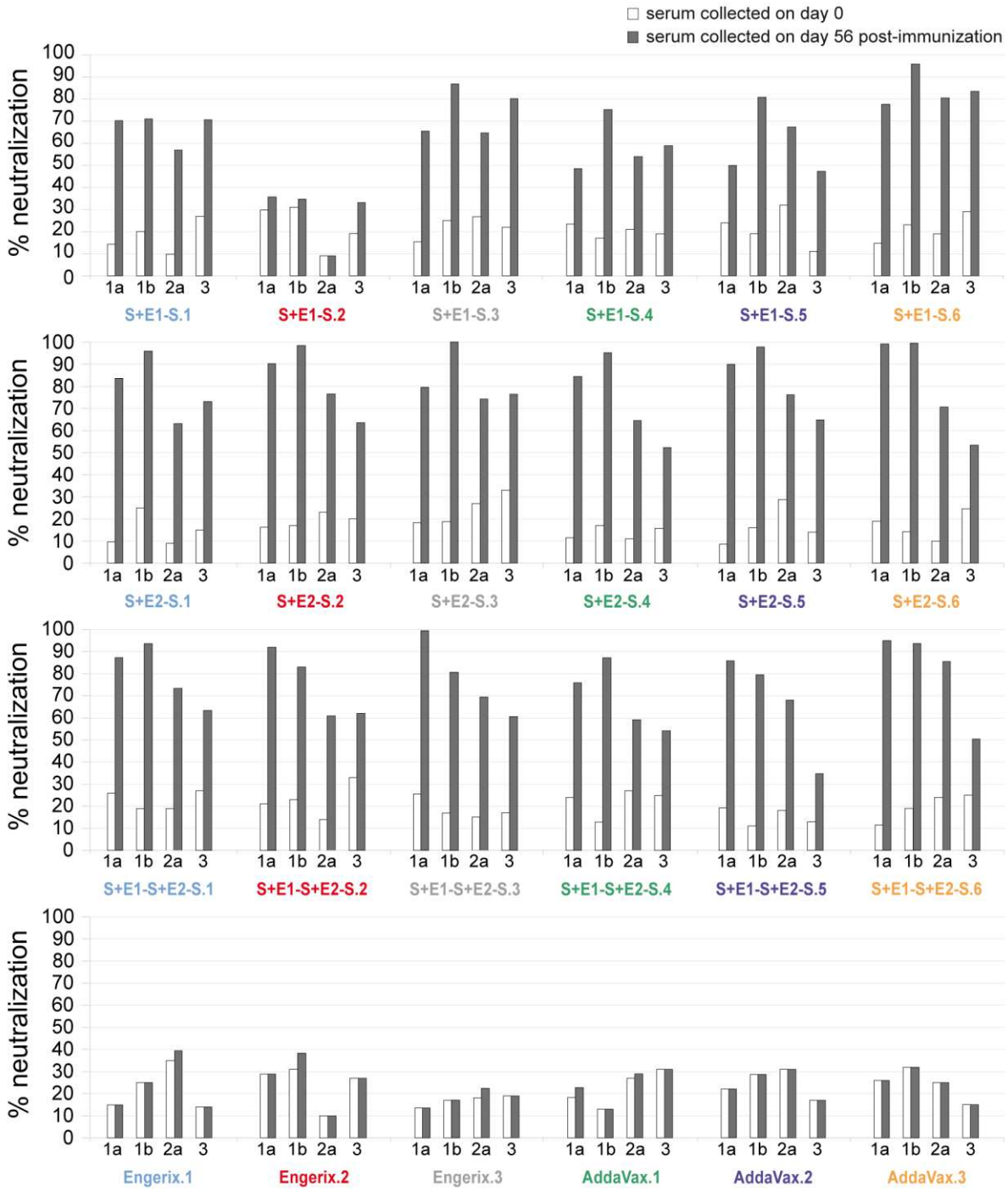



**Fig. 4.** Cross-neutralizing properties of antibodies induced by immunization with chimeric S+E1-S, S+E2-S or S+E1-S+E2-S particles in the presence of AddaVax® adjuvant against HCVcc. HCVcc harboring HCV envelope glycoproteins derived from genotype 1a (JFH1/H77), 1b (JFH1/J4), 2a (JFH1 WT) and 3 (JFH1/S52) isolates were first incubated for 1 hour at 37°C with a 1:5 dilution of rabbit sera collected on days 0 and 56. They were then incubated with Huh7.5 cells for 6 hours. Infection levels were determined after 48 hours of

incubation at 37°C, by an FFU staining assay. FFU were counted under the microscope and the percentage neutralization in the presence of the postimmune serum (D56) was compared with that in the presence of the pre-immune serum (D0) from the same rabbit. The assay was performed in duplicate and the results are expressed as mean values.

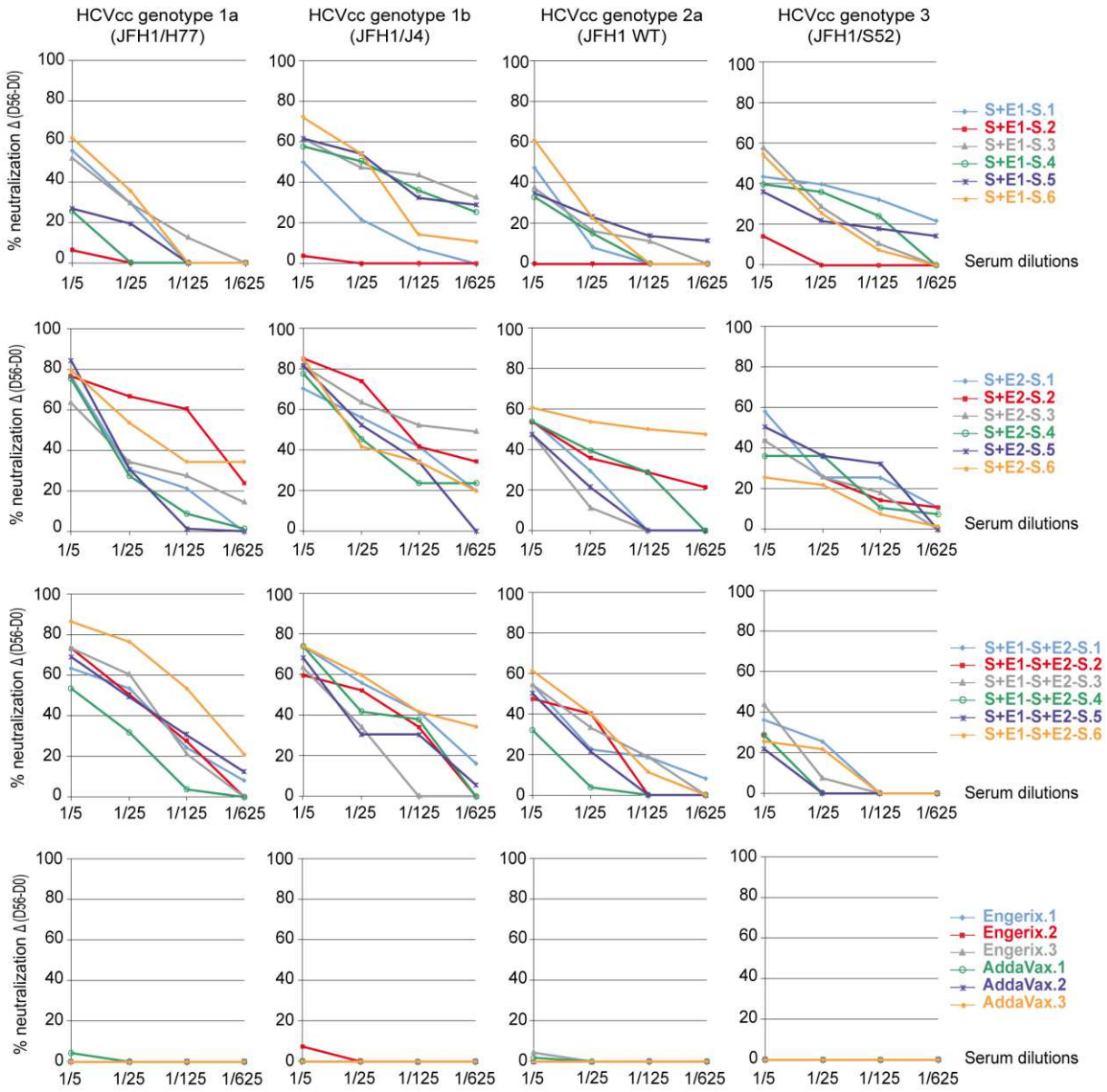



**Fig. 5.** Evaluation of the cross-neutralizing properties against HCVcc of five-fold serial serum dilutions of rabbits immunized with chimeric particles. Dilutions of rabbit sera collected on days 0 and 56 were first incubated with HCVcc harboring HCV envelope glycoproteins derived from genotype 1a (JFH1/H77), 1b (JFH1/J4), 2a (JFH1 WT) and 3 (JFH1/S52) isolates, for 1 h at 37°C, which were then allowed to infect Huh7.5 cells for 6 h. After 48 h of incubation at 37°C, the percentage neutralization was determined by subtracting the infectivity titer obtained with the pre-immune serum (D0) from that obtained with the post-immune serum (D56). The assay was performed in duplicate and the results are expressed as mean values.

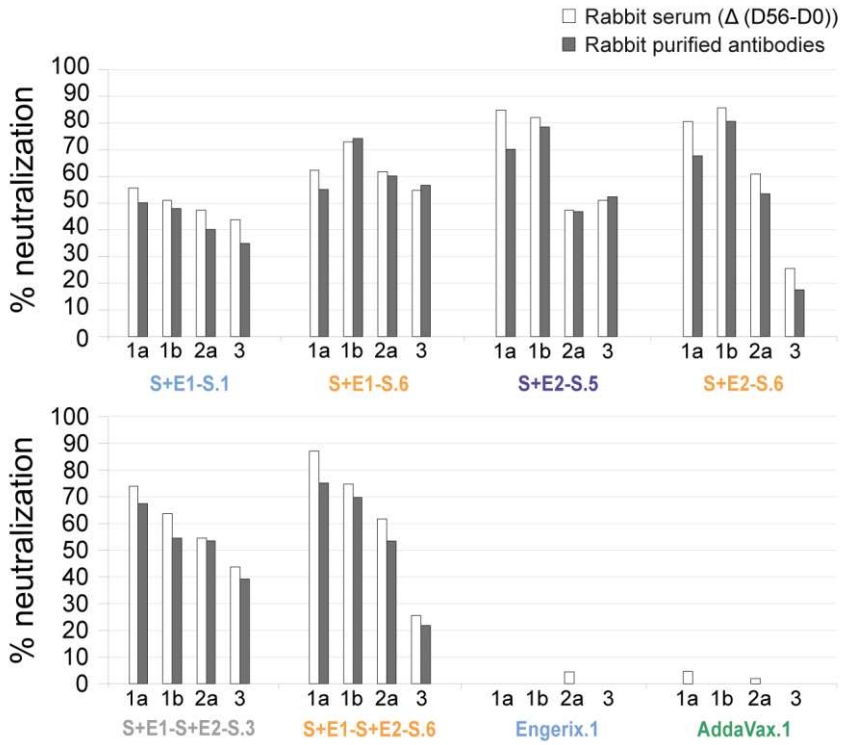



**Fig. 6.** Comparison of the cross-neutralizing properties against HCVcc of sera and antibodies purified from the corresponding sera of rabbits immunized with chimeric particles. Antibodies were purified from rabbit sera with strong neutralizing properties (2 sera for each immunization group) collected on day 56, with the HiTrap protein G HP kit (GE Healthcare) and associated desalting method. HCVcc harboring HCV envelope glycoproteins derived from genotype 1a (JFH1/H77), 1b (JFH1/J4), 2a (JFH1 WT) and 3 (JFH1/S52) isolates were first incubated for 1 hour at 37°C with a 1:5 dilution of purified antibodies or serum. They were then incubated with Huh7.5 cells for 6 hours. Infection levels were determined after 48 hours of incubation at 37°C, in an FFU staining assay. The assay was performed in duplicate and the results are expressed as mean values.

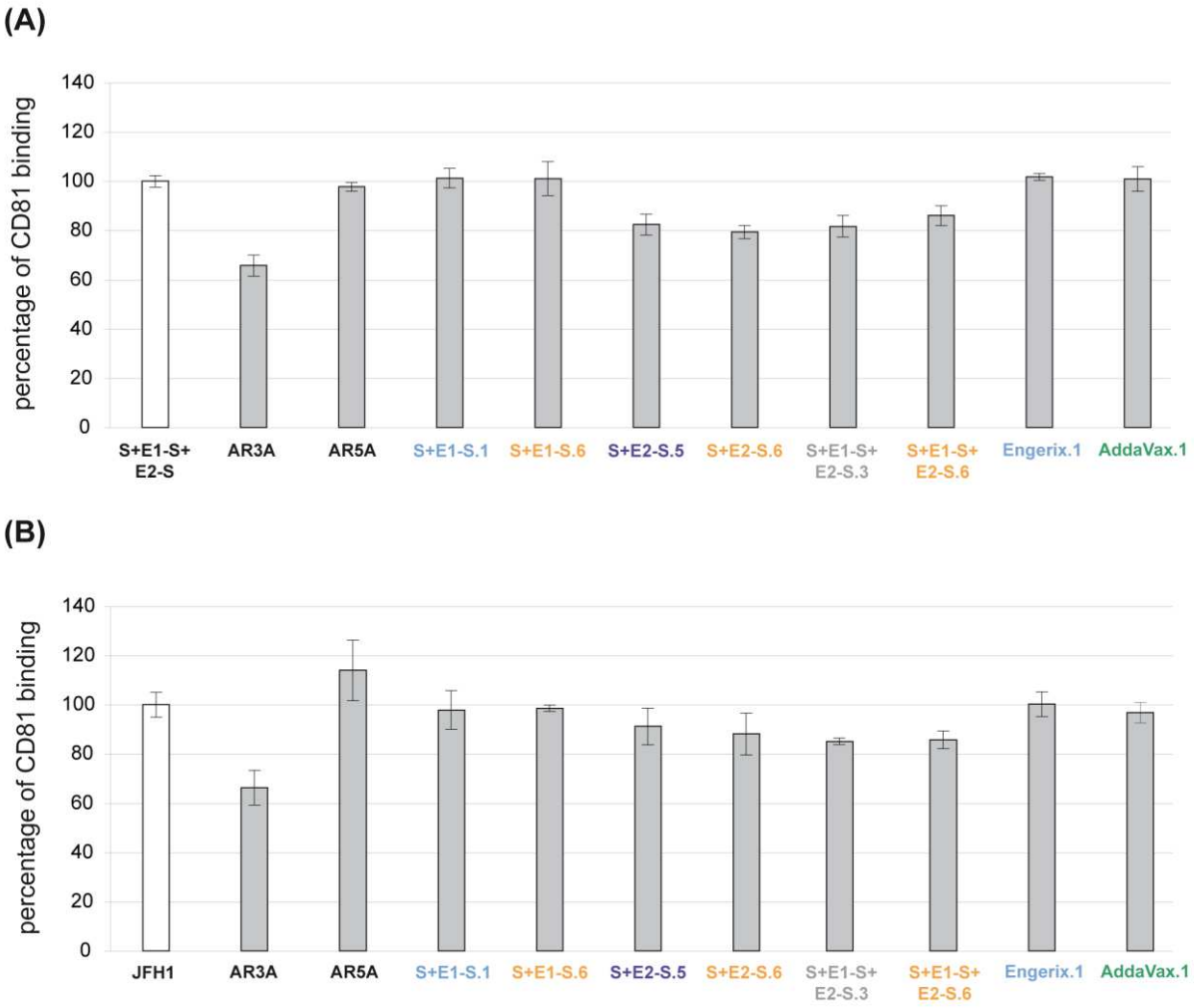



**Fig. 7.** CD81-binding inhibition properties of neutralizing antibodies induced by immunization with chimeric particles. Rabbit sera diluted 1:5 and the human mAbs AR3A and AR5A were incubated with S+E1-S+E2-S chimeric particles **(A)** or purified JFH-1 WT viruses **(B)** for 2 hours at room temperature, before their addition to ELISA wells coated with GST-hCD81. After 2 hours of incubation, the bound complexes were specifically visualized with the human anti-E2 mAb AR3A and a monoclonal peroxidase-conjugated mouse anti-human Ig antibody. Absorbance at 490 nm was determined and compared with that for CD81-binding assays conducted in the absence of mAbs or rabbit serum (100%, white histograms). The final data are expressed as relative binding signals. The data shown are the means ± SD of two independent experiments performed in triplicate.

# Supporting Materials and Methods

***Analysis of anti-E1E2 antibody responses.*** This assay was based on the use of BHK-21 cells producing E1-E2 proteins and control cells expressing β-galactosidase. For recombinant Semliki RNA synthesis, previously described pSFV1-E1E2 and pSFV3-β-Gal constructs (1,2) were linearized by digestion at the single *Spe*I site and the linear plasmids were transcribed *in vitro*, with SP6 RNA polymerase, according to the standard protocol provided by the manufacturer (New England Biolabs). BHK-21 cells ($10^7$) were then electroporated with 10 µg of the various recombinant SFV RNAs, with a single exponential decay pulse at 350 V, 750 µF in a Gene Pulser Xcell (Bio-Rad). Cells were then grown in Glasgow modified Eagle's medium (GMEM; Invitrogen) supplemented with 5% heat-inactivated FCS, 8% tryptose and antibiotics (penicillin and streptomycin). Sixteen hours after transfection, cells were lysed with 1% Triton in PBS and the lysates were clarified by centrifugation at 4°C for 20 min at 12,000 x *g*, assayed for protein concentration and then immediately used for the immunoassay. For anti-E1E2 ELISA, Immulon 2 HB immunoassay plates (ThermoElectron) were coated by incubation overnight at 4°C with a 20 µg/ml solution of lectin from Galanthus Nivalis (Sigma) in PBS. They were washed in PBS and nonspecific binding sites were saturated by incubation for 4 hours at 37°C with 4% sheep serum and 5% milk powder in PBS. The plates were washed with PBS and incubated overnight at 4°C with the E1E2 or β-galactosidase proteins produced in BHK-21 cells (100 µg/ml). The plates were washed with PBS and again blocked by incubation with 4% sheep serum and 5% milk powder in PBS for 4 hours at 37°C. The plates were washed with PBS, rabbit sera were added and the plates were incubated for 2 hours at 37°C. The plates were washed with 0.5% Tween 20 in PBS (PBS-T), and a polyclonal peroxidase-conjugated goat anti-rabbit immunoglobulin antibody (SouthernBiotech) diluted 1:2,000 in PBS was added. The plates were incubated for 1 hour at 37°C, washed with PBS-T, and a mixture of $H_2O_2$ and

*o*-phenylenediamine was added. The plates were left for 15 min at room temperature in the dark, and color development was then stopped by adding 2 N $H_2SO_4$. Absorbance at 490 nm was determined and, for each serum tested, the optic density (OD) value obtained for wells with capture by β-galactosidase protein was subtracted from that obtained for wells with capture by E1E2 proteins. The final data are expressed as the difference in OD (E1E1-β-gal).

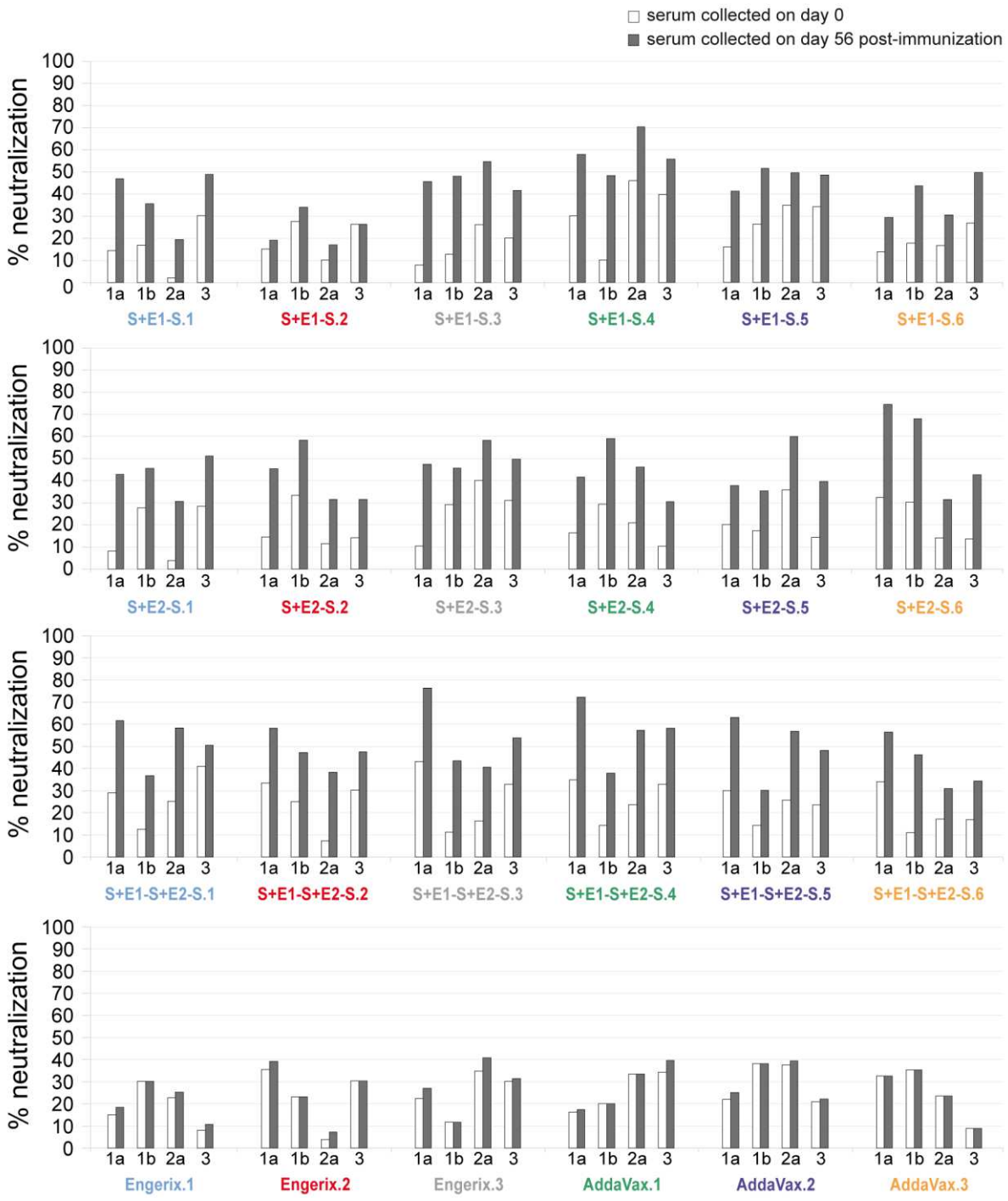
***Neutralization assay with HCVcc.*** HCVcc harboring HCV envelope glycoproteins derived from genotype 1a (JFH1/H77), 1b (JFH1/J4), 2a (JFH1 WT) and 3 (JFH1/S52) isolates (3,4) were used to assess the neutralizing potential of anti-HCV envelope protein antibodies. Viruses were generated by transfecting Huh7.5 cells with RNA, as previously described (5). Cells were then maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics (100 IU of penicillin and 100 µg/ml streptomycin; Invitrogen). Viral supernatants were collected 12 days after transfection, purified by filtration (through a filter with 0.45 $\mu$m pores), and viral infectivity was monitored by infecting $1\times10^5$ Huh7.5 cells with 50 $\mu$l of serial 10-fold dilutions of the viral supernatants in quadruplicate. Infection levels were determined after 48 hours of incubation at 37°C, by a focus-forming unit (FFU) staining assay, with an HCV-positive human serum pool, a biotinylated goat anti-human IgG polyclonal antibody (SouthernBiotech) and horseradish peroxidase-streptavidin reagent (Biolegend). FFU were counted under a microscope and supernatant infectivity titers were determined as the number of FFU/ml. Titrated viral stocks were diluted to obtain 2,000 FFU/ml in growth medium. A volume of 50 $\mu$l, corresponding to 100 FFU, was then incubated for 1 hour at 37 °C with 25 $\mu$l of five-fold serial dilutions of each rabbit serum collected on days 0 and 56. For some experiments, we also used antibodies purified from selected rabbit sera. The virus/serum (or immunoglobulins) mixture was then used to infect 10,000 Huh7.5 cells plated the previous day on 96-well plates (Falcon) for 6

hours at 37°C. The virus-containing medium was then removed, replaced with fresh medium and the infected cells were incubated at 37°C. Infectivity was evaluated 48 hours after infection, as described above. The assay was performed in duplicate and the results are expressed as mean values.

***Neutralization assay with retroviral HCVpp.*** HCVpp harboring HCV envelope glycoproteins derived from genotype 1a (7a) (6), 1b (UKN5.23), 2a (UKN 2a1.2 ) and 3 (UKN3A.1.28) (7) isolates were used to assess the neutralizing potential of anti-HCV envelope protein antibodies. Env-pseudotyped viruses were generated by cotransfecting $3.5\times10^6$ human embryonic kidney 293-T (HEK-293T) cells with 12 $\mu$g of each E1E2 expression construct and 8 $\mu$g of pNL4.3.Luc.R−E− (8), in the presence of calcium phosphate (Invitrogen). Cells were then maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics (100 IU of penicillin and 100 µg/ml streptomycin; Invitrogen). Viral supernatants were collected 72 h later, purified by filtration (filter with 0.45 $\mu$m pores), and viral infectivity was determined by infecting $1\times10^5$ Huh7 cells with 100 $\mu$l of serial five-fold dilutions of the viral supernatants in quadruplicate. Huh7 cells were grown in the medium described above, and infection levels were determined after 72 hours, with the Bright Glo luciferase assay (Promega) and a Centro LB 960 luminometer (Berthold Technologies) for the measurement of luciferase activity in cell lysates. Titrated pseudotyped virus stocks were diluted to obtain 1,000 to 4,000 $TCID_{50}$/ml in growth medium. A volume of 50 $\mu$l, corresponding to 50 to 200 $TCID_{50}$ units was then incubated for 1 hour at 37 °C with 25 $\mu$l of five-fold serial dilutions of each rabbit serum collected on days 0 and 56. For some experiments, we also used antibodies purified from selected rabbit sera. The virus/serum (or immunoglobulins) mixture was then used to infect 10,000 Huh7 cells for 6 hours at 37°C. The virus-containing medium was then removed, replaced with fresh medium

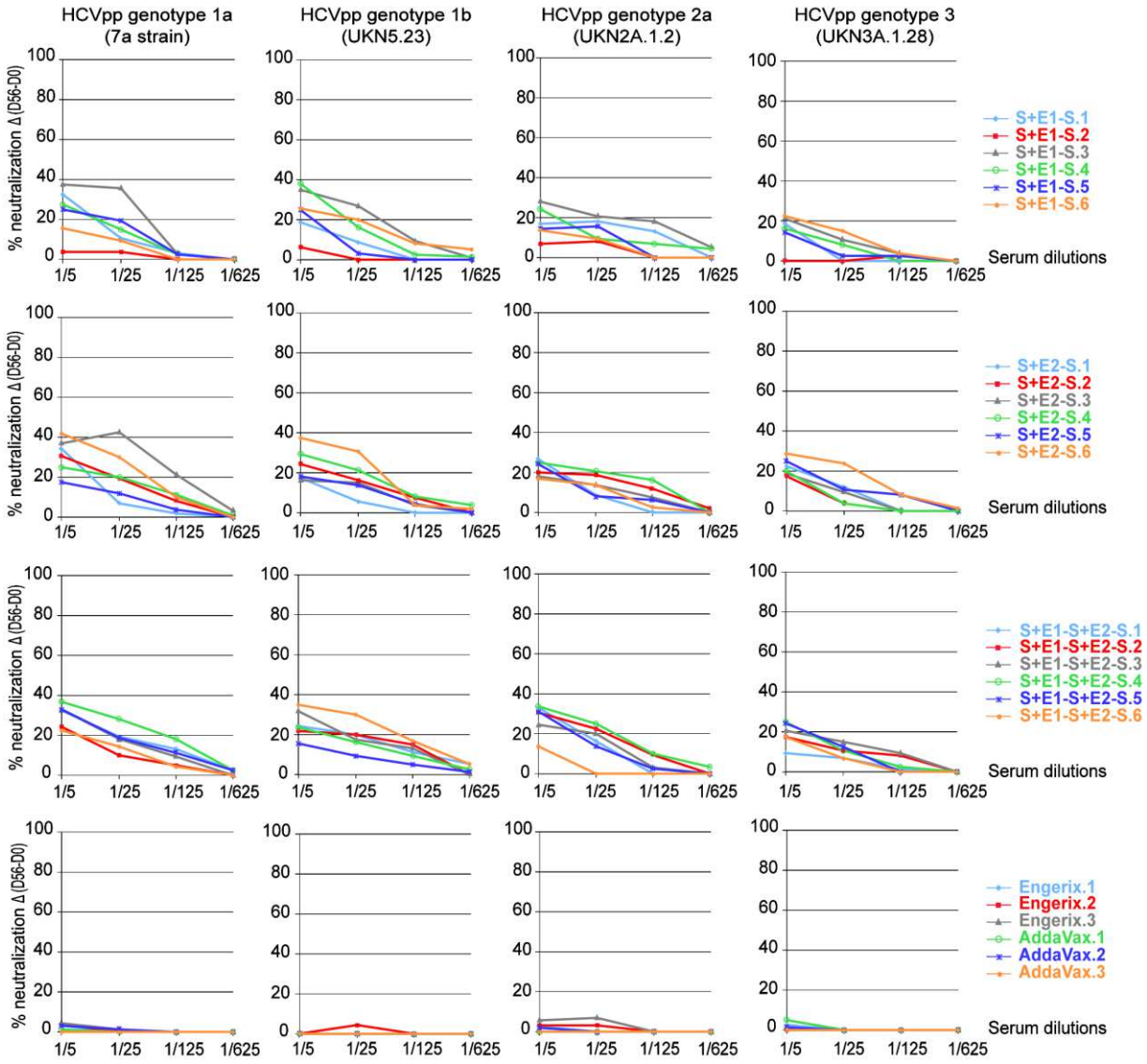
and the infected cells were incubated at 37°C. Luciferase activity was measured 72 hours after infection, as described above. The assay was performed in duplicate and the results are expressed as the mean values.

***CD81-binding inhibition analysis.*** *In vitro* binding assays were conducted to characterize the CD81-binding properties of the chimeric S+E2-S and S+E1-S+E2-S particles and to assess the ability of the NAbs present in rabbit sera to inhibit this interaction. Rabbit sera diluted 1:5 and the human mAbs AR3A and AR5A (10 µg/mL) were incubated for 2 hours at room temperature with S+E1-S+E2-S chimeric particles (25 µg/mL) or JFH-1 WT viruses purified on sucrose cushions (1,000 FFU) before their addition to ELISA wells coated with GST-hCD81 (1 µg/ml; Abnova). After 2 hours of incubation, the bound complexes were specifically visualized with the human anti-E2 mAb AR3A and a monoclonal peroxidase-conjugated mouse anti-human Ig antibody (clone JDC-10) diluted 1:2,000. Absorbance at 490 nm was determined and compared with that for CD81-binding assays conducted in the absence of mAbs or rabbit serum.



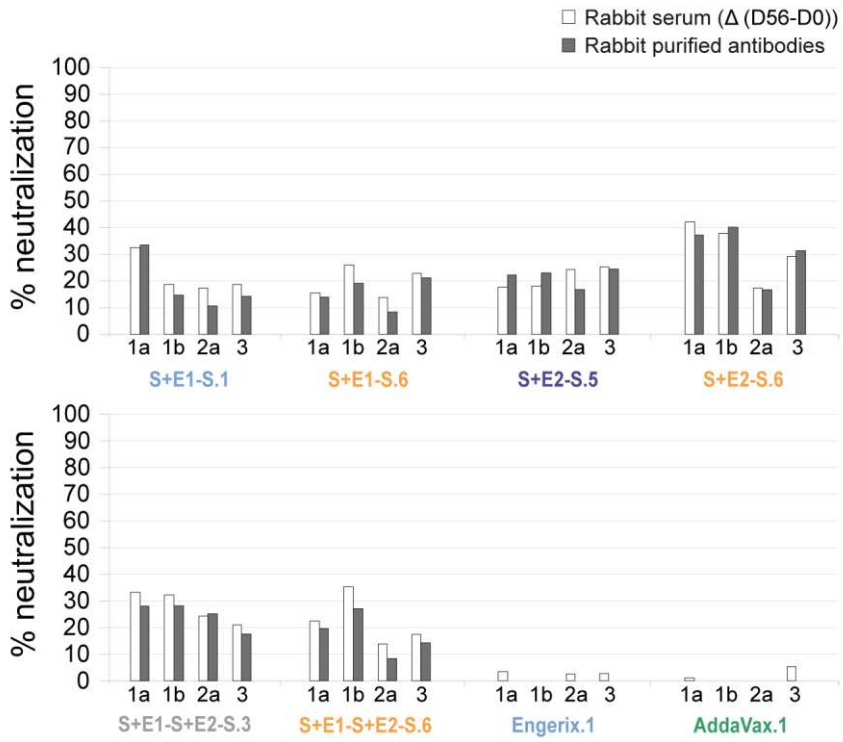

**Supporting Fig. S1.** In the presence of the AddaVax® adjuvant, chimeric S+E1-S, S+E2-S and S+E1-S+E2-S particles elicit antibody responses that cross-neutralize HCVpp. A 1:5 dilution of rabbit sera collected on days 0 and 56 was first incubated for 1 hour at 37°C with HCVpp harboring HCV envelope glycoproteins derived from genotype 1a (7a), 1b (UKN5.23),

2a (UKN 2a1.2) and 3 (UKN3A.1.28) isolates. The mixture was then allowed to infect Huh7 cells for 6 h. After 72 h of incubation at 37°C, the percentage neutralization in the presence of the postimmune serum (D56) was compared with that in the presence of the pre-immune serum (D0) from the same rabbit. The assay was performed in duplicate and the results are expressed as mean values.




**Supporting Fig. S2.** Evaluation of the cross-neutralizing properties against HCVpp of five-fold serial serum dilutions of rabbits immunized with chimeric particles. Dilutions of rabbit sera collected on days 0 and 56 were first incubated with HCVpp harboring HCV envelope glycoproteins derived from genotype 1a (7a), 1b (UKN5.23), 2a (UKN 2a1.2) and 3 (UKN3A.1.28) isolates for 1 h at 37°C, which were then allowed to infect Huh7 cells for 6 h. After 72 h of incubation at 37°C, the percentage neutralization was determined by subtracting the infectious titer obtained with the pre-immune serum (D0) from that obtained with the post-

immune serum (D56) from the same rabbit. The assay was performed in duplicate and the results are expressed as mean values.




**Supporting Fig. S3.** Comparison of the cross-neutralizing properties against HCVpp of sera and antibodies purified from the corresponding sera of rabbits immunized with chimeric particles. Antibodies were purified from rabbit sera selected on the basis of strong neutralizing properties (2 sera for each immunization group) and collected on day 56. HCVpp harboring HCV envelope glycoproteins derived from genotype 1a (7a), 1b (UKN5.23), 2a (UKN 2a1.2) and 3 (UKN3A.1.28) isolates were first incubated for 1 h at 37°C with a 1:5 dilution of purified antibodies or serum. They were then allowed to infect Huh7 cells for 6 h. After 72 h of incubation at 37°C, the percentage neutralization was determined. The assay was performed in duplicate and the results are expressed as mean values.

# Supporting References

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