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Expression, purification and immunogenic characterization of hepatitis C virus recombinant E1E2 protein expressed by *Pichia pastoris* yeast

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ABSTRACT

Development of an effective vaccine may be the key in the control of hepatitis C virus (HCV) infection. Recent studies have shown that HCV envelope proteins can induce broadly neutralizing antibodies against conserved domain for HCV binding to the cellular receptors. So HCV envelope proteins are considered as the major HCV vaccine candidate. In this study, we used *Pichia pastoris* yeast to express truncated HCV E1E2 protein, which consists of E1 residues 187–346 and E2 residues 381–699. The yeast can produce high level of recombinant HCV E1E2 protein. The protein has complex glycosylation and can bind to CD81, the putative HCV receptor. Moreover, the purified protein can efficiently induce anti-E1E2 antibodies in rabbits, which are able to neutralize two kinds of HCV pseudotype particles derived from HCV genotype 1a and 1b, as well as HCV virions derived from HCV genotype 2a. These findings indicate that the recombinant E1E2 glycoprotein is effective in inducing broadly neutralizing antibodies, and is a potent HCV vaccine candidate.

1. Introduction

Hepatitis C virus (HCV) belongs to the *Hepacivirus* genus of the *Flaviviridae* family (Lindenbach and Rice, 2001). HCV infects over 3% of the world population and is the leading cause of chronic liver disease worldwide. The efficacy of hepatitis C treatment is less than satisfactory and only successful in half of the patients (Lauer and Walker, 2001). Development of an effective vaccine may be key in the control of HCV infection.

HCV is an enveloped virus with a single-stranded positive RNA, which encodes the structural proteins E1, E2, core and non-structural proteins (Lindenbach and Rice, 2001). E1 and E2 proteins, two heavily glycosylated enveloped proteins, which can form a heterodimeric complex, are assumed to be exposed on the surface of HCV particle and potentially elicit neutralizing antibodies against HCV infection in the host (Houghton and Abrignani, 2005). Notably, with the development of HCV pseudotype particle (HCVpp) and HCV cell culture (HCVcc) systems (Hsu et al., 2003; Wakita et al., 2005), increasing evidence supports that E1 and E2 proteins are the major vaccine candidates. Some studies have shown that the pivotal amino acids in receptor binding domains of HCV envelope proteins are conserved (Owsianka et al., 2006; Hadlock et al., 2000). HCV envelope proteins can induce broadly neutralizing antibodies against these domains, especially the tetraspanin CD81 binding domain (Keck et al., 2007; Johansson et al., 2007; Stamataki et al., 2007). Moreover, one report shows that E1E2 glycoproteins expressed by mammalian cells can protect chimpanzees from heterogeneous HCV infection (Houghton and Abrignani, 2005). Herein, *Pichia pastoris* yeast was used to explore the possibility of producing an HCV vaccine using envelope proteins in a cheap industrial eukaryotic expression system with high yield. Both monoclonal antibodies (mAbs) against E2 and mAbs against E1 have been reported as broadly neutralizing antibody (Keck et al., 2004, 2007; Johansson et al., 2007). So we constructed a recombinant HCV E1E2 glycoprotein (rE1E2), hoping the rE1E2 can induce neutralizing antibodies more efficiently than E1 or E2 alone. However, the transmembrane domains of E1 and E2 are highly hydrophobic (Lindenbach and Rice, 2001), which probably jeopardize expression. The full-length E1E2 expressed in yeast was hard to be detected with Western blot in our previous study (data not shown). In this study, C-terminally truncated E1 fragment and C-terminally truncated E2 fragment were fused to rE1E2, which was produced at high level in yeast. The rE1E2 has glycans and can bind CD81. More importantly, the rE1E2-induced antibodies are broadly neutralizing antibodies. These data showed the *P. pastoris* yeast-expressed rE1E2 has a promising potential as an HCV vaccine.

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2. Materials and methods

2.1. Antibodies, plasmids, and cell lines

Mouse monoclonal antibody (mAb) A4 against HCV E1 was generously provided by Dr. Jean Dubuisson (Institut de Biologie de Lille, France).

The plasmids pBRTM/HCV 1-3011, pCAGGS/MCS+E1E2 (H77), pCAGGS/MCS+E1E2 (Con1) and pNL4.3.Luc-E$^-$R$^-$ were generously provided by Dr. Charles M. Rice (Rockefeller University, New York, NY). For the genesis of vesicular stomatitis virus (VSV) pseudotype particles (VSVpp), the plasmid pCAGGS+VSV G was constructed with subcloning from pVPack-VSV-G (a generous gift from Dr. Zhengli Shi, Wuhan Institute of Virology, Chinese Academy of Science). The plasmid pJFH1 that contains the full-length HCV genotype 2a JFH1 cDNA downstream of the T7 RNA promoter was kindly provided by Dr. Takaji Wakita (Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan). For creating a HCV reporter virus system, a recombinant plasmid nominated pJFH1-Luc was constructed on the basis of pJFH1 by Yang Wu. A humanized Renilla luciferase gene was fused in frame to the 17 N-terminal residues of the JFH1 core coding region and separated from the HCV open reading frame by a short foot-and-mouth disease virus 2A proteinase cleavable peptide (Yang Wu et al., unpublished data).

The hepatic cell line (Huh7.5.1) was kindly provided by Dr. Francis V. Chisari (Departments of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA). The cell line 293T was purchased from China Center for Type Culture Collection (Wuhan, China).

2.2. Construction of recombinant expression plasmid

The coding regions of E1 (aa187–aa346) and E2 (aa381–aa699) were amplified by PCR from plasmid pBRTM/HCV 1-3011 (strain H77, genotype 1a), using primers E1 forward 1 [5′-GTGCCCGCTTCAGCC-3′], E1 reverse [5′-CTGCAGGTCCATGATGGCTTG-3′] (Pst I), E2 forward [5′-TCCGACTGCAGGCGGAAACCCACGTC-3′] (Pst I), and E2 reverse 1 [5′-TCCGAAAGCTT**CTA**CACGTCCACAATGTT-3′] (Hind III) (restriction sites are underlined and the reverse stop codon **CTA** is bold). The E1 amplified product was ligated into pMD18-T vector (Takara), resulting in pMD18-T-E1. The E2 amplified product was cloned into pMD18-T-E1 between the Pst I and Hind III sites to create pMD18-T-E1E2. The recombinant E1E2 DNA fragment was linked by Pst I site (CTGCAG, resulting in Leu Gln after translation) in pMD18-T-E1E2, then amplified by PCR using primers E1 forward 2 [5′-TCCGATACGTAGTGCCCGCTTCAGCC-3′] (SnaB I) and E2 reverse 2 [5′-AAGCTTGCGGCCG**CTA**CACGTCCACAATGTTC-3′] (Not I), and then cloned into pPIC9K vector (Invitrogen), resulting in a recombinant plasmid nominated pPIC9K-E1E2.

The large extracellular loop (LEL, aa113–aa202) domain of human CD81 was amplified by PCR from plasmid pMal-c2.2/4-LEL (a generous gift from Dr. Shoshana Levy, Stanford University, Stanford, CA), using primers LEL forward [5′-TCTCTGGATCCTTTGTCAACAAGGACCAG-3′] (BamH I), and LEL reverse [5′TGTGTAAGC**TTA**GAGCTTCCCGGAGAAGAG-3′] (Hind III). The amplified fragment was then cloned into pET-GST vector (Gene Power Laboratory Limited), resulting in pET-GST-LEL. All clones were confirmed by DNA sequencing.

2.3. Expression and purification of rE1E2

rE1E2 was expressed by *P. pastoris* yeast according to the manufacturer's manual (Multi-Copy Pichia Expression Kit, Invitrogen). Briefly, pPIC9K-E1E2 was linearized by Pme I and electroporated into *P. pastoris* strain SMD1168. His$^+$ Mut$^+$ SMD1168 transformants were selected on Minimal Dextrose (MD) medium plate and confirmed on Minimal Methanol (MM) medium plate. Multiple inserted recombinants were isolated on Yeast Extract Peptone Dextrose (YPD) medium plate containing G418 at final concentration of 2.0 mg/ml. A single colony of multiple inserted His$^+$ Mut$^+$ SMD1168 recombinants was inoculated into 20 ml YPD in a 100 ml baffled flask, and grew at 29 °C in a shaking incubator (300 rpm) until culture reached an OD_{600} = 6. The cells were harvested by centrifuging at 3000 × *g* for 5 min and resuspended to an OD_{600} of approximately 1 in 150 ml MM in a 500 ml baffled flask. The flask was covered with 2 layers of sterile gauze and returned to incubator to continue growth. To the culture was added methanol to a final concentration of 0.5% every 24 h to maintain induction. The samples were collected every 12 h and analyzed by SDS-PAGE and Western blot. After 72 h culture, the cells were collected and stored at −70 °C. For extraction and purification of rE1E2, the cells were disrupted by glass beads in TEN buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, and 150 mM NaCl) and then centrifuged. The rE1E2 mainly existed in the deposit with cell fractions, and was not soluble in TEN buffer. The deposit was resuspended in TEN buffer with 8 M urea and centrifuged again. The supernatant was dialyzed against 5 mM Tris–HCl (pH 9.3) overnight at 4 °C and then clarified by centrifugation at 12,000 × *g* for 30 min. The supernate was first loaded to a Q-Sepharose Fast Flow column with 20 mM Tris–HCl (pH 9.3). After washing with 0.05 M NaCl and 20 mM Tris–HCl (pH 9.3), the rE1E2 was eluted with 0.5 M NaCl and 20 mM Tris–HCl (pH 9.3). Then the eluent was loaded to a Phenyl Sepharose Fast Flow column with 20 mM Tris–HCl (pH 9.3) and 0.1 M NaCl. The rE1E2 was eluted with 20 mM Tris–HCl (pH 9.3). The rE1E2 was further purified with Sephadex G150 column for removing low molecular weight fractions. The purified rE1E2 was dialyzed against 5 mM Tris–HCl (pH 9.3) and sterilized with 0.45 μm filter, and then stored at 4 °C.

2.4. Animals, immunization and ELISA

Approximately 600 μl of the rE1E2 (300 μg) diluted in 2 ml sterilized 0.9% NaCl was used for immunization. Two 4-month old female New Zealand rabbits were provided from the Experimental Animals Center, Wuhan University (Wuhan, China) and were raised in the conditions of Good Laboratory Practice (GLP). The rabbits were subcutaneously immunized in multiple sites on the back with 0 μg (negative control) or 300 μg purified rE1E2, respectively. Booster injections were given with the same dose at 2, 3, 4, 5 and 13 weeks later. Sera were obtained every week. The anti-rE1E2 titers in sera were measured by ELISA as previously described (Han et al., 2006). Microtiter plates were prepared by coating each well with 100 ng purified E1E2 recombinant glycoprotein. Antibody titers against rE1E2 were estimated as the maximum dilution that in ELISA gave for the immunized serum an A_{492} value above duplication of the negative control serum A_{492} values.

2.5. Endoglycosidase digestion assay

Purified rE1E2 was digested with endo-β-N-acetylglucosaminidase H (endo H), or peptide: N-glycosidase F (PNGase F) as described previously (Op De Beeck et al., 2004). The digested proteins were treated according to the manufacturer's instructions (New England Biolabs) and then analyzed by Western blot with mAb A4.

2.6. CD81 pull-down assay

Glutathione S-transferase (GST)-CD81 LEL fusion protein (GST-LEL) was generated in *Escherichia coli* BL21 (DE3) plysS strain according to the guideline of Gene Rapid High Expression Kit (Gene

Power Laboratory Limited). GST-LEL or GST proteins were released from *E. coli* cells by sonication and then purified by glutathione-Sepharose 4B column (GE healthcare).

Glutathione-Sepharose 4B beads bound GST-CD81 LEL or GST protein (approximately 150 μg protein) was prepared in 100 μl of PBS. The beads were then mixed with 200 μl of rE1E2 (60 μg protein) and 800 μl of PBS. The mixture was rotated for 2 h at 4 °C. After washing 5 times with ice-cold PBS, the rE1E2 attached on the beads was separated by SDS-PAGE, and then analyzed by Western blot assay as previously described (Li et al., 2007). The antibodies-specific bands were detected by enhanced chemiluminescence (Pierce) and exposed to X-ray film (Fuji).

2.7. HCVpp genesis, infection and neutralizing assay

Pseudoviruses were generated as previously described (Hsu et al., 2003). Briefly, 293T cells were cotransfected with an E1E2 expression plasmid and pNL4.3.Luc.R$^-$E$^-$, using Lipofectamine 2000 (Invitrogen). Lipid–DNA complexes were removed 6 h later and replaced with DMEM supplemented with 3% FBS. Supernatants were harvested at 48 or 72 h after transfection, pooled and filtered. A commercially available HIV type 1 p24 antigen ELISA protocol (Biomérieux) was used to quantify the p24 content of samples.

For infection, Huh7.5.1 cells were seeded at 8000 cells per well of a 96-well plate the day prior to infection. The medium was removed and pseudotype virus stock, diluted in DMEM supplemented with 3% FBS and 4 μg/ml of Polybrene (Sigma), was added. After overnight incubation, the inoculum was removed and replaced with DMEM supplemented with 3% FBS. At 72 h postinfection, the medium was removed and the cells were lysed with 40 μl of cell lysis buffer (Promega) per well. Luciferase activity was assayed by the addition of 35 μl of lysate to 50 μl of luciferase substrate and measured as luciferase relative light units (RLU) in luminometer.

For neutralizing assay, sera were heat-inactivated at 56 °C for 1 h, mixed with HCVpp or VSVpp in DMEM supplemented with 3% FBS at their appropriate dilution (1:100, 1:300 and 1:1000) and incubated at 37 °C for 1 h. The virus–serum mixture was transferred to Huh-7.5.1 cells as above described. The percent of neutralization of serum was determined by comparing viral infectivity in the presence of post-immune serum to the mean infectivity in the presence of pre-immune serum at the same dilution. The data are from the mean of four replicate wells.

2.8. HCVcc genesis, infection and neutralizing assay

For generating infectious HCV reporter virus (JFH1-Luc), in vitro transcribed JFH1-Luc genomic RNA was transfected into Huh7.5.1 cells as described (Wakita et al., 2005). Cell culture media collected at 10 day post-transfection were centrifuged and passed through 0.22 μm filter. The cell-free media were used as virus stocks, which were aliquoted and stored at −80 °C. Cell supernatants were serially diluted 10-fold in DMEM supplemented with 10% FBS and used to infect 8000 naive Huh-7.5.1 cells per well in 96-well plates. The inoculum was incubated with cells for 1 h at 37 °C and then added with DMEM supplemented with 10% FBS. The level of HCV infection was determined at 72 h postinfection by immunofluorescence staining for HCV Core. The viral titer is expressed as focus forming units per milliliter of supernatant (ffu/ml), determined by the average number of core-positive foci detected at the highest dilutions.

Intracellular staining was performed as described (Zhong et al., 2005). Monoclonal anti-core mouse antibody (Abcam, ab2740) was used at a dilution of 1:1000 followed by incubation with a 1:1000 dilution of FITC-conjugated goat anti-rabbit IgG (pierce) for 1 h at room temperature.

For neutralization assays, the heat-inactivated sera were diluted at an appropriate dilution (1:20, 1:100, 1:500) and pre-incubated with 5000 ffu of JFH1-Luc virus in a volume of 100 μl for 1 h at 37 °C. The virus–serum mixture was then used to infect 8000 Huh-7.5.1 cells in a 96-well plate for 6 h at 37 °C. The virus-containing medium was removed, replaced with DMEM with 10% FBS and incubated at 37 °C. The efficiency of the infection was measured as RLU in luminometer at 72 h postinfection as same as HCVpp. The percent of neutralization of serum was determined by comparing the number of RLU in the presence of post-immune serum to the average number of RLU in the presence of pre-immune serum at the same dilution. The data are from the means for four replicate wells.

3. Results

3.1. Expression and purification of rE1E2

In this study, the truncated HCV E1 and E2 coding regions were ligated and integrated into the chromosome of SMD 1168 yeast strain and under the control of alcohol oxidase promoter. The yeast cells produced high level of rE1E2 after induction by methanol for 48 h (Fig. 1A). According to the absorption at 280 nm of purified rE1E2, the final yield of rE1E2 was estimated at 35 mg/l. After cell disruption, rE1E2 mainly existed in the deposit with cell fractions, showing that most of expressed rE1E2 was not secretive. The reason might be that most of rE1E2 aggregated in the endoplasmic reticulum. Western blot assay showed that there were four forms of rE1E2: 72, 95, 145 and over 200 kDa (Fig. 1B). The 72 and 95 kDa proteins might be the two basic different glycosylated forms. The 145 kDa protein might be the dimer of 72 kDa protein as judged by their molecular sizes. Over 200 kDa protein was the aggregate (Agg), which was also observed by SDS-PAGE when E1E2 was expressed in mammalian cells (Op De Beeck et al., 2004).

After purification, 95 kDa protein and over 200 kDa protein could be detected by Western blot (Fig. 1D), and only 200 kDa could be observed by SDS-PAGE (Fig. 1C). The disappearance of 95 kDa band in SDS-PAGE analysis after purification (Fig. 1C) should be due to self-assembling of 95 kDa protein or loss in purification process. The purity of the final purified rE1E2 is estimated as >95% according to SDS-PAGE analysis.

3.2. Characterization of glycosylation on rE1E2

Endo H removes the chitobiose core of high mannose oligosaccharides and some hybrid forms of N-linked sugars, but not the complex forms (Op De Beeck et al., 2004). After denaturing, purified rE1E2 was either untreated or digested with endo H or PNGase F as a deglycosylation control that removes all types of N-linked glycans. Two bands were detected after endo H digestion (Fig. 2, lane 2), showing high level of mannose oligosaccharides in the E1E2 protein (Fig. 2, comparing lane 1 to lane 2). The faster-migrating band was completely deglycosylated, as was the band that represented complete deglycosylation after PNGase F treatment (Fig. 2, comparing lane 2 to lane 3). The slower-migrating one disappeared after PNGase F treatment, suggesting that this form contained complex forms of N-linked glycans (Fig. 2, comparing lane 2 to lane 3).

3.3. rE1E2 can bind to CD81

As a putative HCV receptor, CD81 is able to bind HCV E2 glycoprotein, HCVpp and HCV virion (Hadlock et al., 2000; Lambot et al., 2002; Cocquerel et al., 2003; Pileri et al., 1998). The LEL motif of CD81 is responsible for the binding (Zhang et al., 2004). CD81 pull-down assay showed that rE1E2 could bind to GST-CD81 LEL protein (Fig. 3A). Studies by others showed that exposure of HCV

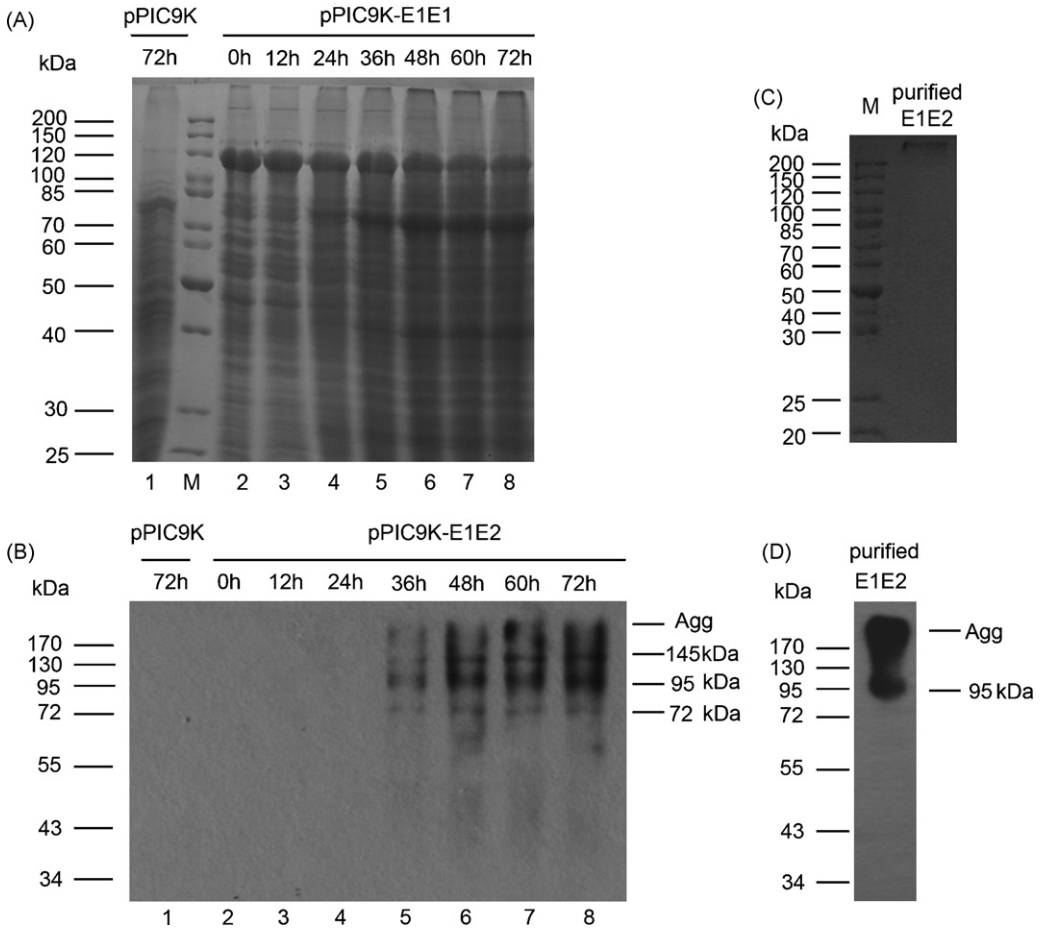

Fig. 1. Analysis of E1E2 proteins that are expressed by *pichia pastoris* yeast SMD1168. The samples were collected at indicated timepoints post-inducing (line 2–8) and then were analyzed by SDS-PAGE (A) and by Western blot with anti-HCV E1 mAb A4 (B). The yeast cells harboring pPIC9K were used as a negative control (line 1). rE1E2 aggregates (Agg) are indicated. The purified E1E2 protein was analyzed by SDS-PAGE (C) and by Western blot with mAb A4 (D). The SDS-PAGE gels were stained by Coomassie brilliant blue R-250.

virions to acidic pH buffer (pH 5.5) followed by return to neutral pH did not affect HCV infectivity (Hsu et al., 2003; Tscherne et al., 2006). Similarly, rE1E2 could bind to GST-CD81 LEL protein even when pretreated with acidic buffer (pH 5.5) (Fig. 3B).

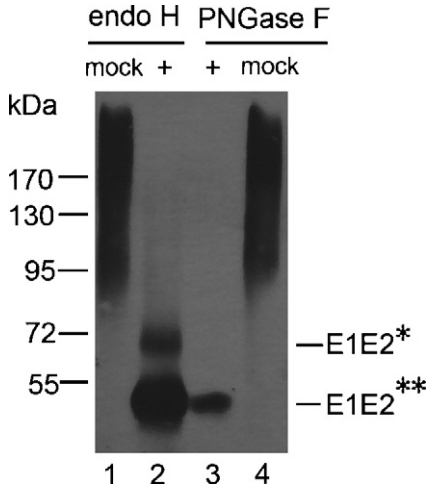

Fig. 2. Analysis of the glycans associated with HCV E1E2 glycoprotein. The purified E1E2 glycoprotein was treated with or without endo H (lane 1 and lane 2) or PNGase F (lane 3 and lane 4) for 24 h at 37 °C and then analyzed by Western blot with mAb A4. The endo H-resistant form of rE1E2 was indicated by one asterisk. Two asterisks indicated the deglycosylated form of rE1E2.

3.4. Characterization of humoral response induced by rE1E2 in rabbit

Purified rE1E2 was used to immunize rabbit without any adjuvant. We followed seroconversion of two female rabbits immunized with or without rE1E2. Immunized rabbit seroconverted at 2 weeks after primary immunization. After booster injections, anti-

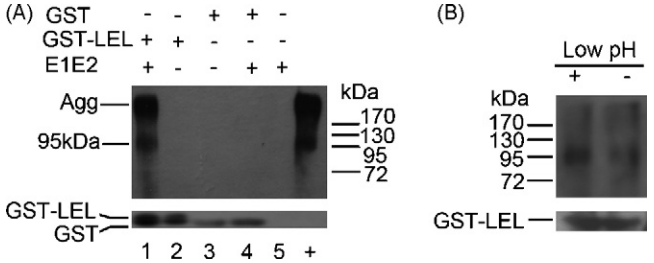

Fig. 3. The interaction between rE1E2 and CD81. (A) GST-CD81-pull-down assay. rE1E2 with GST-LEL (lane 1), GST-LEL protein alone (lane 2), GST protein alone (lane 3), rE1E2 with GST (lane 4), and rE1E2 alone (lane 5) were precipitated with beads. rE1E2 without beads was used as a positive control in Western blot (lane +). (B) Effect of low-pH treatment on binding of rE1E2 to CD81. The low-pH treatment was carried out as previously described (Hsu et al., 2003). Briefly, rE1E2 was treated with MES (pH 5.5) for 20 min at 37 °C. After the pH was adjusted to neutral, rE1E2 was applied to CD81-pull-down assay. rE1E2 was detected by Western blot with mAb A4. GST-LEL and GST were detected by Western blot with goat anti-GST antibody (GE Healthcare). The molecular sizes (kDa) are indicated in the panel.

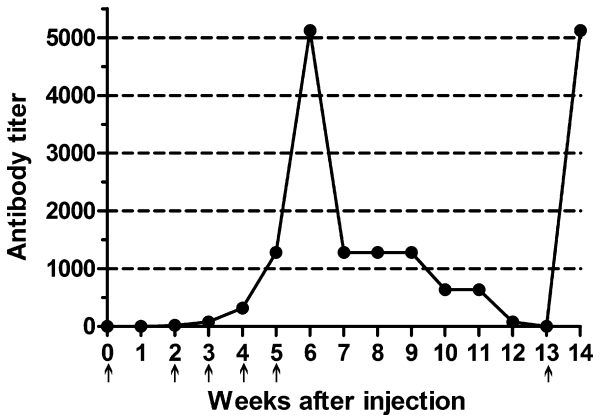

Fig. 4. Time course of antibody response to rE1E2 in immunized rabbit. Rabbits were injected with 0 μg (negative control) or 300 μg rE1E2 without adjuvant. Arrows indicated the time of immunization. Results represented the value of anti-E1E2 antibody titers of immunized rabbit. The titers were defined as the maximum reciprocal serum dilution able to recognize rE1E2 above duplication of negative control serum A_{492} values in ELISA.

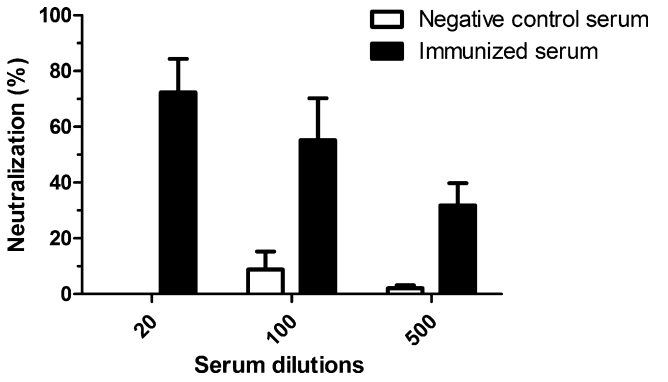

Fig. 6. The immunized rabbit serum neutralized HCVcc. 5000 ffu HCV JFH1-Luc virus infected 8000 Huh7.5.1 cells in the presence of sera for 6 h. The HCV infectivity was measured as RLU in luminometer at 72 h postinfection. The percent of neutralization of serum was determined by comparing viral infectivity in the presence of post-immune serum to the mean infectivity in the presence of pre-immune serum at the same dilution. Bars represent the mean of four replicate wells and the error bars represent S.E.M. value.

body titers increased, up to 1:5120 after 5 weeks (Fig. 4). Then the antibody titers decreased to below 1:20 at 13 weeks later. After booster injection at 13 weeks later, antibody titers increased up to 1:5120 again at 14 weeks later (Fig. 4).

3.5. rE1E2 can induce antibodies capable of neutralizing HCVpp and HCVcc infectivity

To assess whether the E1E2-specific responses measured by ELISA indicated broadly neutralizing antibodies, sera from immunized and control rabbits were screened for their ability to neutralize HCVpp-H77 or HCVpp-Con1 infectivity, respectively. The results showed that the serum from immunized rabbit could neutralize HCVpp derived from HCV genotype 1a (H77) or 1b (Con1) in a dose-dependent manner (Fig. 5). The serum could not neutralize VSVpp, which indicated the neutralizing ability of immunized serum was HCV-specific.

Entry studies using HCVpp and HCVcc have yielded remarkably convergent results, but some features may differ (Tscherne et al., 2006). Besides, both of HCVpp-H77 and HCVpp-Con1 are derived from HCV genotype 1, and HCVcc-JFH1-Luc is derived from HCV genotype 2. For these reasons, HCVcc neutralizing assay was employed to confirm the broadly neutralizing ability of the immunized serum. The data showed the immunized serum could neutralize HCVcc (genotype 2a) in a dose-dependent manner (Fig. 6).

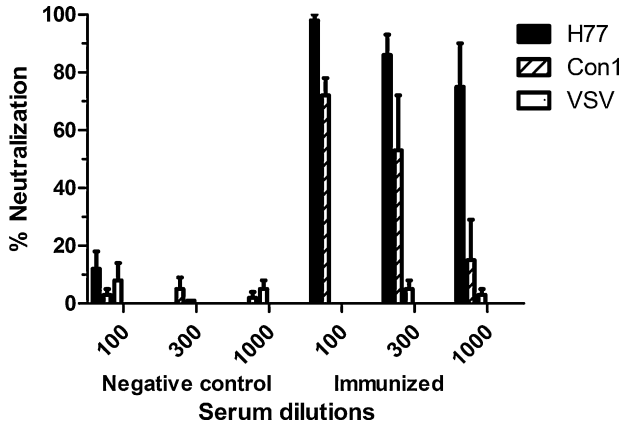

Fig. 5. The rE1E2 elicited antibody responses that neutralized HCVpp infectivity. Sera at dilutions of 1:100, 1:300 and 1:1000 were pre-incubated with HCVpp expressing diverse envelope glycoproteins and VSVpp (control) for 1 h at 37 °C and allowed to infect Huh-7.5.1 cells for 6 h. Infection was terminated at 72 h and infectivity determined (luciferase activity). Percent neutralization is determined by comparing infectivity of HCVpp-H77 (black), Con1 (diagonal stripes) and VSVpp (white) in the presence of post-immune sera to the infectivity in the presence of pre-immune sera at the same dilution. Bars represent the mean of four replicate wells and the error bars represent S.E.M. value.

4. Discussion

One of the urgent goals of HCV research is to develop an effective vaccine. Lots of studies have shown that the HCV envelope proteins can induce broadly neutralizing antibodies, making them as the major candidate for HCV vaccine (Houghton and Abrignani, 2005; Keck et al., 2007; Johansson et al., 2007). Although one study showed that it was difficult to express HCV envelope proteins by *P. pastoris* yeast (Martinez-Donato et al., 2006), we expressed high level of HCV rE1E2 by this system (Fig. 1). The discrepancy might be the truncated and fused E1E2 proteins used in our study as well as the different yeast strains used for expression and the expressed rE1E2 was easily purified by chromatography for industrial purpose. The purified rE1E2 comprised mainly of high molecular weight aggregate and does not represent the native E1/E2 heterodimer (Fig. 1C). Because of high variability of HCV E2 and deficiency of epitope data of anti-E2 mAb, we cannot obtain a mAb against E2 recognizing rE1E2. However, the fact that the rE1E2 can bind to CD81 is a proof of rE1E2 consisting of E1 and E2, because E1 alone cannot bind to CD81. In addition, rabbit polyclonal antibodies induced by truncated E2 protein expressed in bacteria can recognize purified rE1E2 (data not shown).

The glycans of HCV envelope proteins are very important to their structures, functions and antigenicity (Helle et al., 2007; Deleersnyder et al., 1997; Falkowska et al., 2007). Therefore, it is indispensable to use eukaryotic expression system to express HCV envelope glycoproteins. *P. pastoris* yeast can synthesize and process complex glycoprotein (Morel and Massoulié, 1997). In this study, rE1E2 had glycans and some glycans of rE1E2 exhibited endo H resistance, which could be digested by PNGase F (Fig. 2, comparing lane 2 to lane 3). This is similar to some glycans of native HCV envelope glycoproteins (Op De Beeck et al., 2004; Flint et al., 2004). Some glycans of rE1E2 should be different to native E1E2 because of the difference between yeast and mammalian cells. The differences among glycans in this study apparently do not affect the ability of binding CD81 and inducing broadly neutralizing antibodies in

animals, which are important to the goal of the study. The rE1E2 exhibited a stable CD81 binding ability, even when pretreated with low-pH buffer (Fig. 2), showing that yeast-expressed rE1E2 should be correctly folded in CD81 binding domain. The CD81 binding domain of rE1E2 probably induced broadly neutralizing antibodies against this domain, as the rE1E2-immunized serum neutralized HCVpp infectivity and HCVcc efficiently (Figs. 5 and 6). There are also other possibilities, including that the broadly neutralizing antibodies are due to the presence of linear epitopes within E2 or E1 and/or due to the presence of a low level of native E1/E2 in the preparation.

Many human monoclonal antibodies against HCV E1 or E2 are confirmed as broadly neutralizing antibodies (Hadlock et al., 2000; Keck et al., 2007; Johansson et al., 2007; Broering et al., 2009). Some human monoclonal antibodies have the potential to prevent HCV reinfection in liver transplant patients (Schiano et al., 2006; Eren et al., 2006). However, broadly neutralizing antibodies are rarely obtained in human combinatorial libraries (Johansson et al., 2007). Therefore, the strategy of vaccination with HCV E1E2 glycoprotein for inducing broadly neutralizing antibodies is probably successful to control HCV as it succeeds in chimpanzees (Houghton and Abrignani, 2005). In this study, rE1E2 can induce high humoral immune response (Fig. 4) that neutralize HCVpp infectivity and HCVcc efficiently (Figs. 5 and 6) as do HCV envelope glycoproteins expressed by mammalian cells do (Stamataki et al., 2007). The comparison of the antibodies induced by rE1E2 with E1/E2 expressed in mammalian cells should be important. Future study would be performed to compare the antigenicity of rE1E2 with E1/E2 expressed in mammalian cells plus adjuvant. Moreover, *P. pastoris* yeast is an efficient and convenient eukaryotic expression system. We believe that the *P. pastoris* yeast-expressed rE1E2 is a promising HCV vaccine candidate for industrial purpose.

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