

# Human antibodies from phage display libraries: expression of recombinant full length immunoglobulin G specific to the Hepatitis C virus E2 glycoprotein

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## SUMMARY

Evidence from clinical and experimental studies indicates that hepatitis C virus E2 glycoprotein (HCV/E2) represents a major target antigen involved in the containment and resolution of naturally occurring HCV infection. Antibody phage display allows the molecular cloning of cDNA sequences encoding antibody fragments specific to a wide range of diverse antigens. These antibodies may be produced in bacteria as Fab or converted into full length IgG. The latter have a higher serum half life and display Fc encoded function. Using a library prepared from an HCV-infected individual, we selected a panel of Fab fragments for binding to invariant epitopes of the E2 glycoprotein. This work describes a technique used to convert the selected Fab fragments into full length IgG and to express these antibodies in eukaryotic cells. All the recombinant antibodies retained the binding specificity of the parental Fab showing an increase in apparent relative affinity for E2.

**KEY WORDS:** Phage display, Human recombinant antibody, Mammalian expression vectors, Hepatitis C virus

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## INTRODUCTION

Hepatitis C virus (HCV) infection has become one of the world's most important health problems and is currently the most frequent cause of blood-borne and community-acquired non-A non-B hepatitis (Choo *et al.*, 1989; Kuo *et al.*, 1989). In a large proportion of the infected population (70-80%) the virus persists after primary infection, with a significant risk of progression to cirrhosis of the liver and hepatocellular carcinoma (Alter *et*

*al.*, 1992; Perico *et al.*, 2009). End-stage liver disease secondary to HCV infection accounts for approximately 50% of liver transplants in the United States and in Europe. It has been estimated that in the USA alone more than four million individuals are infected with HCV and that between 2010 and 2019 these infections will cost as much as \$10.7 billion in direct medical expenditures (Wong *et al.*, 2000; Rosen, 2001; Curry, 2004; Irshad *et al.*, 2008).

Despite great efforts, the research community has failed to identify a target antigen, allowing successful vaccination of primates. As a result our current ability to control the infectious cycle is extremely limited.

An accumulating body of evidence from clinical and animal studies suggests that a B cell mediated immune response may confer at least partial protection against HCV primary infection and

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persistence (Farci *et al.*, 1992; Choo *et al.*, 1994; Farci *et al.*, 1994; Shimizu *et al.*, 1994). If this is so, neutralizing antibodies may play a role in the natural resolution and containment of HCV infection. In particular, two viral gene products, the E1 and E2 envelope glycoproteins, have been shown to represent major targets for protective immune responses (Rollier *et al.*, 2004; Zampino *et al.*, 2004). Chimpanzees induced to produce antibody against E1 or E2 glycoproteins showed increased resistance to HCV infection, mainly due to an anti-E2 humoral immune response (Abrignani *et al.*, 1998; Montserrat *et al.*, 2004). In humans, HCV reactive immunoglobulins have been shown to be effective in prophylaxis for infection by the virus (Piazza *et al.*, 1997). In vitro, antibodies against E2 can prevent E2/CD81 interaction, a key step in HCV infection (Pileri *et al.*, 1998; Allander *et al.*, 2000; Dubuisson *et al.*, 2002; Dubuisson *et al.* 2007).

In culture, these antibodies prevent the binding of E2 to cells expressing CD81 (Rosa *et al.*, 1996; Cocquerel *et al.*, 2006). As a result, they are often called neutralization of binding (NOB) antibodies. High titres of NOB antibodies have been associated with the natural resolution of HCV infection in humans (Ishii *et al.*, 1998). HCV does not replicate efficiently in cultured cells. This problem has hampered systematic assessment of the ability of NOB antibodies to prevent virus infection and replication.

Today, however, phage display techniques make it possible to rapidly generate human monoclonal antibodies with desired specificities. In work reported elsewhere, we used this technique to dissect the antibody response of an HCV-positive patient, generating a large panel of human monoclonal antibody fragments specific for E2 and with a remarkable diversity in NOB activity and epitome (Burioni *et al.*, 1998; Bugli *et al.*, 2001; Burioni *et al.*, 2001).

We then used a cross strain selection strategy to select Fabs directed against invariant E2 epitopes. These antibodies could potentially confer protection against a wide range of viral strains and are less likely to drive selection of HCV escape variants.

However, in some cases Fabs might have fewer effects in immunoprophylaxis than glycosylated full length antibodies. In post exposure prophylaxis for HCV, it is essential to maintain a high

titre of circulating antibodies. In humans, glycosylated full length antibodies have a longer serum half life than Fabs. It therefore seems likely that passive immunization with glycosylated, full length antibodies, could provide a higher level of protection against HCV infection.

Further advantages of full length IgG include higher avidity, due to their dimeric epitope binding sites and the presence of important immunological functions encoded by the Fc domain (Meyer *et al.*, 2002). In view of these considerations, an efficient method for producing glycosylated full length antibodies could be extremely useful.

This work explored one possible method, using the VH/VK express eukaryotic expression vector system (Persic *et al.*, 1997) to convert E2 specific Fab fragments into full length IgG. Four different human Fab fragments were chosen to be converted into whole immunoglobulins: e8, e20, e137 and e509. While affinity of the different antibody fragments appeared similar, activity in inhibiting E2 binding to target cells varied considerably from one Fab fragment to another (Burioni *et al.*, 1998). The variable region of heavy chain gene (isotype IgG1) and the variable region of k chain gene of each antibody clone inserted into the phage display vector pCombIII were separately amplified and subcloned in mammalian expression vectors.

The recombinant full length IgG derived from those four Fab fragments, purified from CHO culture supernatant, retained the full activity of the parental Fabs, binding specifically to HCV E2 glycoprotein with an apparent increase in relative binding affinity to E2 antigen.

## MATERIALS AND METHODS

### *Basic molecular biology and cell culture techniques*

Rt-PCR amplification reactions were performed under standard conditions according to manufacturers' instructions, using Superscript II as reverse transcriptase (Invitrogen,) and a low error rate Pfu DNA polymerase (Invitrogen).

Restriction endonuclease reactions, ligation agarose gel electrophoresis and all general subcloning procedures were performed following established protocols unless the product manu-

facturer suggested otherwise. DNA sequencing was performed on a 373A automated DNA sequencer (Perkin Elmer, Norwalk CT) using a Taq fluorescent dideoxy terminator cycle sequencing kit (Perkin Elmer). Cell lines were cultured under sterile conditions at 37°C, 5% CO<sub>2</sub>, in a humidified incubator (Forma scientific). Cell lines were maintained in DMEM supplemented with 10% foetal calf serum (Eurobio). For large scale (0.5-1 L) production of recombinant protein, cells were adapted for growth in serum free, protein free culture medium (HyQ PF CHO, Hyclone).

#### *Subcloning of the variable regions from anti E2 Fabs into VH/VKexpress vectors*

In previous work we had created a panel of human antibody fragments specific for E2 glycoprotein from an IgG1/Kappa phage display library from patient with a high serum titre of anti-HCV. To obtain this panel the human antibody fragment library had been screened using a E2 antigen derived from a viral strain (genotype 1a) which was different from the strain (genotype 1b) infecting the patient from whom the library was originally obtained.

This strategy selected for cross-reactive antibodies, directed against conserved regions of envelope glycoprotein, known to exhibit little sequence variation among different viral strain. Fabs e8, e20, e137 and e509 selected from the library were all able to bind E2 glycoprotein of genotype 1a with high affinity. We assembled full length IgG cDNAs by amplifying the cDNA sequences encoding the variable regions of the heavy and light chain from each parental pCombIII clone. PCR products were then subcloned into the VH and VK express mammalian expression vectors.

The VHexpress and VKexpress plasmids constitute a mammalian expression system specifically developed (Persic *et al.*, 1997) to assemble antibody fragments from combinatorial phage display libraries into full length IgG and drive their recombinant expression in a mammalian host. The vector system is made up of a number of functional cassettes flanked by unique restriction sites.

The two vectors encode two different eukaryotic selectable markers (neomycin and mycophenolic acid), allowing direct selection of double transfectants. Subcloning relied on two conserved re-

striction sites on the 3' end of the human VH and VK sequences (CellIII, and XhoI respectively) and on restriction sites in frame to the plasmid encoded secretory leader sequence at the 5' end. These latter restriction sites were introduced via the oligonucleotide primers used to amplify the parental Fab. The sequences for the relevant oligonucleotides were as follows:

VH_Fwd (PstI)	ATTGCTGCAGCAGTCTGGG GCTGAGGTGAAG
VH_Rev (CellIII)	TATAGCGCTGAGCTCTCAC CAACTTTCTTGTCCACCTTG GTG
VK_Fwd (SacI)	AGGGAGCTCACGCAGTCTC CAGCCACCC
VK_Rev (XhoI)	CCTGTCTGGAACCTCGAGCG GCCCCGGGAGG

#### *Expression of anti E2 VH and VK constructs in BHK 293 cells*

The day before transfection, BHK 293 cells were plated on 60mm dishes at 60% confluence in complete DMEM 10% FCS. Cells were transfected using the CaPO<sub>4</sub> method following instructions provided by the manufacturer (Calcium Phosphate transfection kit, Invitrogen). 48 h post transfection cell culture medium was assayed for antibody production.

#### *Generation of a stable CHO cell line expressing anti E2 VH and VK construct*

Chinese hamster ovary (CHO) cell lines were used for stable transfection. CHO cells were cultured at 37°C with 5% CO<sub>2</sub>, and 95% air, under humidified conditions. Cells were grown and maintained in DMEM supplemented with 10% foetal calf serum (Eurobio).

Separate heavy (VH-express) and light (VK-express) chain vectors were introduced into CHO cells by Polybrene-mediated transfection followed by DMSO osmotic shock (Persic *et al.*, 1997). Stable clones were selected using both G-418 (0.7 mg/ml) and mycophenolic acid (10 µg/ml). To ensure high volume production of protein, we used CHO cells adapted for growth in serum-free, protein free medium (HyQ PF CHO, Hyclone).

*Affinity purification of recombinant full length IgG*  
For each of the clones, 750 ml of conditioned HyQ PF CHO medium were loaded onto an anti-human IgG column (goat anti-human Fab,

Sigma, crosslinked with DMP, Sigma onto protG-sepharose, Pharmacia), washed with 50 ml of PBS buffer and eluted with 10 ml of citric acid (pH 2.3). The eluted fractions were immediately neutralized with 100  $\mu$ l of 1 M Tris, pH 8. The purity of the monoclonal antibody preparations was determined by reducing and non-reducing SDS-PAGE analysis, followed by Comassie blue staining.

#### *Antibody titration and relative affinity determination*

ELISA was performed using the methods described in (Sambrook *et al.*, 1989). Briefly, ELISA plates (Costar) were coated overnight with E2 (Abbot, purified recombinant E21a expressed in insect cells by Baculovirus infection) (25  $\mu$ l, 10  $\mu$ g/ml) at 4°C using carbonate buffer at pH 8.5. Plates were washed and blocked with 1% BSA in PBS for 1 hour at 37°C.

Fifty  $\mu$ l of purified IgG preparation was added (50 ng/ml) and incubated for 2 hours at 37°C. Plates were washed 10 times with PBS/0.05% Tween 20 (Sigma).

50  $\mu$ l of horseradish peroxidase conjugated goat anti-human IgG Fc specific secondary antibody (Sigma) was added to each plate (10<sup>-4</sup> dilution in PBS/1% BSA). After 1 hour at 37°C, plates were washed as above and 100  $\mu$ l of DAB H<sub>2</sub>O<sub>2</sub> solution (Sigma tablets) was added. After 30 minutes at room temperature, plates were read at 450 nm (ABS).

Data was computed by averaging four consecutive readings (Williamson *et al.*, 1993). As a negative control, all the human recombinant antibody preparations were tested against wells coated with BSA only, under the same conditions. Determination of the relative affinity of the full length IgG was performed as described in ref (Burton *et al.*, 1991 and Lesniewski *et al.*, 1995). Briefly, human purified immunoglobulin, at a concentration corresponding to 50% of maximum binding, were tested in ELISA in competition with different concentrations of the same E2 antigen.

IgG and antigen were added to the plate and incubated for 3 hours at 37°C. Antibody relative affinity was computed from the E2 concentration achieving 50% inhibition of antibody binding, as described in ref. (Rath *et al.*, 1988; Burton *et al.*, 1991 and Lesniewski *et al.*, 1995).

## RESULTS

### *Subcloning of the VH and VK sequences into the VH/VK-express vector system*

The combinatorial phage display library approach to antibody repertoire cloning recently made it possible to isolate and produce large quantities of recombinant human Fabs binding with high affinity to specific antigens (Burioni *et al.*, 2001; Bugli *et al.*, 2004).

We previously selected and characterized a panel of human antibody fragments specific for E2 glycoprotein from an IgG1/Kappa library from patient with a high serum titre of anti-HCV antibodies.

Here we describe the assembly of synthetic expression units encoding full length human anti-E2 antibodies and the efficient expression of these constructs in mammalian cell lines.

The VHexpress and VKexpress plasmids constitute a mammalian expression system specifically developed (Persic *et al.*, 1997) to assemble antibody fragments derived from combinatorial phage display libraries into full length IgG and drive their recombinant expression in a mammalian host.

Transcription of the IgG transcript units is controlled by the strong ubiquitously active EF-1 $\alpha$  promoter and the human heavy and light chain constant regions and modified Ig secretory leaders peptide are also encoded in the expression cassette as separate exons. Two different eukaryotic selectable markers (neomycin and mycophenolic acid) are encoded the two vectors, allowing direct selection of double transfectants. Using the vector system described in the methods section, we converted four antibody fragments from the phage display library (Fab e8, e20, e137 and e509) into full length human IgG antibodies (f-e8, f-e20, f-e137 and f-e509). The DNA fragments encoding the Fab VH and VL domains were amplified by PCR from the pCombIII constructs, and subcloned into the VH and VK vectors. The new recombinant vectors were sequenced to verify the 5' cloning frame and the correct assembly of the 3' splice site.

### *Expression of full length anti E2 IgG in mammalian cells*

Expression of IgG from the recombinant plasmids was tested by transient co-transfection of

VH and VK constructs in 293 HEK cells. Forty-eight hours post transfection the medium was collected and assayed for the presence of antibody binding to E2 coated microtitre plates (data not shown).

All four couples of vectors were able to drive efficient expression of full length IgG specifically binding to the parental E2 antigen and recognized by an anti-Fc secondary antibody. We observed no reactivity against unrelated antigens (BSA).

As a next step, stable cell lines were generated by co-transfection of separate VH and VK vectors into CHO cells.

Three days post transfection, cells were plated at low density and grown under double selection in DMEM medium containing G-418 and mycophenolic acid. After 10 days, single colonies were individually picked and grown in 24-well culture plates.

Several clones were assayed for antibody production by ELISA. Those showing the highest antibody titre in the culture supernatant and with the best growing capability were further expanded and processed for long-term storage under liquid nitrogen.

To ensure high volume production of recombinant antibody, transfected CHO cells were previously adapted for growth in serum-free, protein-free medium (HyQ PF CHO medium). After several iterations of this procedure, all the CHO clones showed optimal growth in protein free medium. We then proceeded to expand and seed the clones in large T125 cell culture flasks.

#### *Purification and binding activity of the anti E2 full length IgG*

Antibodies were purified by affinity chromatography on a human specific anti-Fc sepharose column, starting from 0.75 litre of conditioned medium. Different antibodies were expressed at different level.

After purification, antibody yields were in the 800-1200 µg/L range. Each of the antibodies was analysed by SDS-PAGE under reducing and non-reducing conditions.

As shown in figure 1, all the antibodies appear to be correctly assembled and glycosylated. In all cases the electrophoretic mobility of the recombinant antibody was indistinguishable from that of a commercial human reference IgG. These results are consistent with the presence of an H2L2 heterotetramer.

There was no evidence for aberrant antibody species assembly or for any imbalance in the stoichiometry of heavy and light chain production. In all cases the results showed a canonical pattern with light and heavy polypeptide chains present in equimolar amounts (Fig. 1).

To compare the binding affinity of the recombinant IgG to that of the parental Fabs we performed an inhibition ELISA essay using purified E2 protein as the competitor.

We first used titration on E2 coated plates to determine the concentration of purified full length antibody required to achieve binding to 60% of the maximum (OD<sub>450</sub>). Each antibody was then mixed with free E2 glycoprotein in the ELISA wells at a concentration ranging from 10<sup>-7</sup> to 10<sup>-11</sup> M [19].

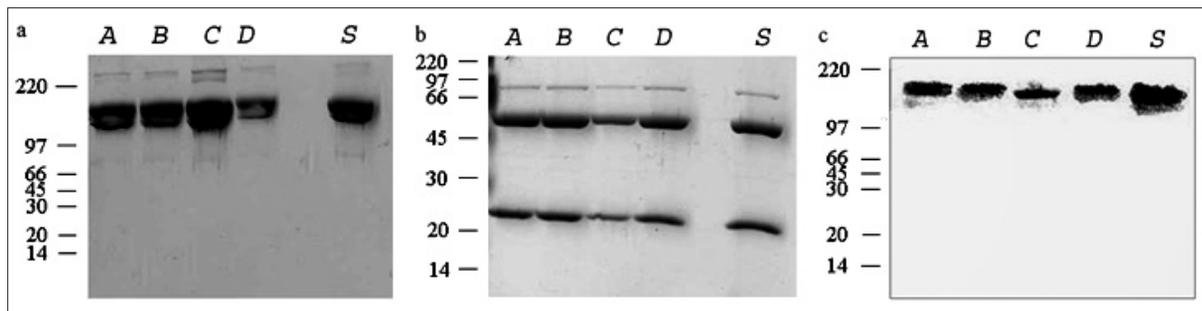


FIGURE 1 - *Comassie blue staining and Immunoblot of the recombinant antibodies purified from CHO cells culture supernatant. The recombinant antibodies were separated by SDS-PAGE (10% PAA) under nonreducing (a) and reducing (b) conditions. Following transfer to nitrocellulose membranes (c), antibody chains were detected with a HRP-conjugated goat anti-human (Fc specific) antibody. A human IgG sample was used for reference under non-reducing conditions. A=e8, B=e20, C=e137, D=e50 are four different full length IgG; S=commercial human monoclonal antibody.*

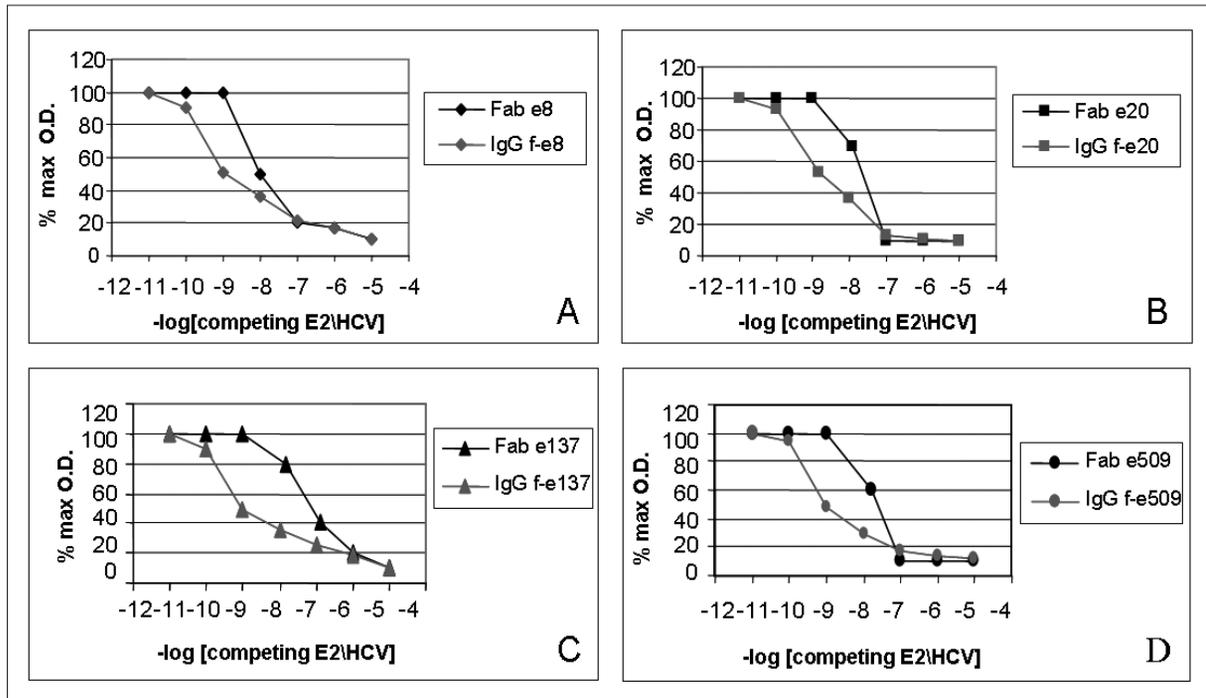


FIGURE 2 - Determination of relative binding affinities of the purified anti-E2 recombinant antibodies (A=e8, B=e20, C=e137, D=e509). In the figure, the relative binding affinity of the full length IgG (red line) and that of the parental Fab fragment (black line) are shown. Relative binding affinities were estimated by inhibition ELISA. Affinities are expressed as percentages of maximum  $OD_{450}$

As shown in Figure 2, the antigen  $IC_{50}$  for the full length immunoglobulin ranged from  $10^9$  to  $10^{10}$  mol/L<sup>-1</sup>; about 10 times higher than the E2  $IC_{50}$  of the parental Fab ( $10^7$  to  $10^8$  mol/L<sup>-1</sup>, figure 2). Recombinant mAbs, purified from CHO culture supernatant, retained the full activity of the parental Fab, binding specifically to HCV E2 glycoprotein with an apparent increase in E2 relative binding affinity.

## DISCUSSION

This paper described a technique used to express a panel of recombinant human Fab fragments with high HCV NOB activity as full length IgG in a mammalian host.

The resulting product is a full length immunoglobulin with the same specificity as the parental Fab fragment and with no apparent loss in  $IC_{50}$  and therefore in binding affinity. The procedure is rapid, straightforward and well-suited for general application. There is evidence that passive transfer of HCV immune IgG may effec-

tively protect primates against recent infection by targeting the E2 and E1 glycoprotein viral antigens (Farci *et al.*, 1996; Lanford *et al.*, 2001). This suggests that full length IgG could become a useful prophylactic, preventing HCV infection in individuals whose risk of infection is higher than that of the general population.

The potential beneficiaries include liver transplant patients (Dickson, 1998; Markowitz *et al.*, 1998; Iacobellis *et al.*, 2008) patients under dialysis (Agarwal *et al.* 2009), individuals accidentally exposed to infected material and individuals who engage in high-risk behaviour.

Full length IgG has a long serum half-life, avoiding the need for repeated administration, as would be required by anti-HCV Fab (Ames *et al.*, 1995; Prabhu *et al.*, 2004).

Its dimeric structure leads to a localised increase in the concentration of epitope binding structures, thereby increasing the probability of reassociation after dissociation.

This produces tighter binding to the antigen and a lower rate of dissociation and could improve its effectiveness in neutralizing HCV. A further

advantage over the use of recombinant Fabs may result from IgG's Fc domain encoded effector functions which have been shown to play a role in the inhibition of HCV infection, in vitro (Meyer *et al.*, 2002).

Last but not least the strategy originally used for selecting the human Fabs was optimized to select for antibodies cross-reactive among different viral strains. IgGs derived from these antibodies could therefore afford protection against HCV, even in cases where there is no prior knowledge of the specific viral strain to which the patient has been exposed.

The procedure described in this paper was very successful resulting in the production of new anti HCV immunoglobulin. As the parental antibody fragments specific for E2 shown a remarkable heterogeneity when assayed by determination of NOB activity, we can hypothesize that recombinant full IgG derived from those Fab fragments preserve the same neutralizing activity.

However the role of these molecules in interfering the binding of E2 to its cellular target requires further study. Moreover, our data suggest that the antibodies produced could be cross-reactive among different viral strains.

Obviously more accurate experiments must be performed on primary isolates to draw any conclusions on E2 cross-reactivity. To conclude, human antibody fragments cloned from phage display libraries may be conveniently converted into full length IgG and expressed with high efficiency by the use of the VH/VKexpress antibody expression system. The application of the procedure described will effectively result in the production of full length immunoglobulin maintaining the specificity of the parental Fab fragment without any evident loss in binding affinity or specificity.

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