Combinatorial library-based profiling of the antibody response against hepatitis C virus in humans

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The antibody response plays a crucial role against hepatitis C virus (HCV) infection, and our understanding of this intricate progress in vivo is far from complete. We previously reported a novel and robust technique based on a large combinatorial viral antigen library displayed on the surface of the yeast Saccharomyces cerevisiae, allowing comprehensive profiling of polyclonal antibody responses in vivo in both qualitative and quantitative terms. Here, we report the generation and application of a combinatorial library of HCV strain JFH1 envelope glycoprotein to profile the antibody response in four HCV chronically infected individuals. By systematic analysis of the location and frequency of antigenic fragments along the JFH1 envelope glycoprotein, we showed that the major binding antibody response was targeted to E2 (80.9–99.8 %), whilst that against E1 was relatively small (0.3–19.0 %). A total of five major antigenic domains (D1–D5) were identified: one was within E1 and an additional four within E2, despite substantial variability among the different individuals. However, serum absorption with the yeast clones containing the antigenic domain D1 resulted in more reduction in neutralizing antibody activity against pseudotyped HCV than those in E2, suggesting that E1 contains additional neutralizing epitopes. Our results have provided additional insights into the HCV-specific antibody response in humans and should assist in a better understanding of protective antibody immunity and in guiding the development of effective vaccines and therapeutics against HCV infection.

INTRODUCTION

Hepatitis C virus (HCV), a member of the family Flaviviridae, is the major aetiological agent of chronic hepatitis. As in many other viral infections, the antibody response against HCV plays an important role in protection against initial infection and disease progression (Burke & Cox, 2010; Di Lorenzo et al., 2011; Edwards et al., 2012; Farci et al., 1994; Kaplan et al., 2003; Law et al., 2008; Morin et al., 2012; Neumann-Haefelin & Thimme, 2013; Osburn et al., 2010; Ploss & Evans, 2012; Tarr et al., 2012; Troesch et al., 2006; Wahid & Dubuisson, 2013; Wang et al., 2011). Individuals who are able to clear HCV during acute infection have strong cellular immune responses as well as autologous neutralizing antibodies during the early phase of infection (Heim, 2013; Logvinoff et al., 2004; Neumann-Haefelin & Thimme, 2013; Pestka et al., 2007; Rehermann, 2009). Whilst other mechanisms exist, the major mechanism of action of neutralizing antibodies is to interfere with or disrupt the interaction between the HCV envelope glycoprotein and the cellular receptor (Burke & Cox, 2010; Di Lorenzo et al., 2011; Edwards et al., 2012; Farci et al., 1994; Giang et al., 2012; Hsu et al., 2003; Johansson et al., 2007; Kaplan et al., 2003; Morin et al., 2012; Neumann-Haefelin & Thimme, 2013; Osburn et al., 2010; Ploss & Evans, 2012; Tarr et al., 2012; Troesch et al., 2006; Wahid & Dubuisson, 2013; Wang et al., 2011), such as scavenger receptor class B type I (SR-BI), the tetraspanin protein CD81 and the tight-junction proteins claudin-1 (CLDN1) and occludin (OCLN) (Edwards et al., 2012; Giang et al., 2012; Haberstroh et al., 2008; Johansson et al., 2007; Moradpour et al., 2007; Rice, 2011; Wahid & Dubuisson, 2013; Wang et al., 2011).

Our previous knowledge on the antibody response against HCV is based largely on mAbs isolated from either experimental animals or artificial human antibody libraries.

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One supplementary table and two figures are available with the online Supplementary Material.
As many of the animal-derived mAbs may be unique from the species perspective, and artificial human antibodies are derived from non-native paired genes for light and heavy chains, their relevance to the true nature of the antibody response in humans is unclear. Ideally, the best approach to study human antibody responses would be to use human body fluids such as serum or plasma to dissect out which antigenic domains are recognized and what proportion of the overall response is attributable to each antigenic domain. Alternatively, one could directly clone and characterize as many paired antibody genes as possible from single B-cells of infected individuals to infer the polyclonal antibody response in vivo. Recently, attempts have been made to use random peptide library fragments and single B-cell-derived antibodies to probe the polyclonal response in vivo (Kachko et al., 2011; Scheid et al., 2009; Wilson & Andrews, 2012; Wrammert et al., 2008; Wu et al., 2010). However, as peptide fragments are restricted by length and lack of post-translational modifications, and the polyclonal antibody response is far more complex than mAbs can account for, neither of these approaches can provide sufficient scope or specificity on the actual antigenic domains recognized by the complex polyclonal antibody response. Thus, as routine or elegant as they are, these available measures remain inadequate.

We previously reported a novel and robust technique to comprehensively characterize the polyclonal antibody response in vivo (Zuo et al., 2011). The technique was based on yeast surface display of a combinatorial library of the viral antigen of interest and has the following unique advantages. First, the combinatorial antigen library displayed on the yeast surface closely resembles those antigens expressed in eukaryotic cells with post-translational modification such as glycosylation and extensive disulfide isomerization, both absent in prokaryotes. Secondly, it can express both linear and conformational epitopes that are significantly more in quantity compared with those used in ELISA or other protein-based detection approaches. Thirdly, the production of antigens is reliable and cost-effective through repeated cycles of induction of the yeast library. This avoids the time-consuming steps required for protein production and purification. Fourthly, as staining and detection of antigens is conducted in solution by FACS, the antigen displayed on the yeast surface and selected by the polyclonal serum will most likely reflect the intrinsic interaction between antigen and antibody within the host. This feature will minimize the confounding non-specific interaction of polyclonal antibodies with solid surface-bound antigens used in ELISA, biopanning in phage display or other assays. Lastly, the antigens displayed on the yeast surface can potentially be used to identify the functional domains of the target protein for use as an initial scaffold for rational vaccine design and development.

In this report, we have applied this technique to characterize the antibody response against the envelope glycoprotein of HCV strain JFH1 in chronically infected individuals. Through systemic analysis of selected antigenic sequences and neutralization assays, we found that the E1 envelope protein contained additional neutralizing epitopes whilst E2 contained epitopes that were largely recognized by the binding antibodies. We believe that our study has provided unique insights into the protective antibody immunity against HCV and should assist our effort in developing effective vaccines and therapeutics against HCV infection.

RESULTS

Construction and validation of a combinatorial JFH1 E1E2 antigen library displayed on the surface of the yeast

We constructed a combinatorial library of full-length envelope from JFH1, the first infectious HCV molecular clone, isolated initially from a fulminant hepatitis patient in Japan (Wakita et al., 2005), and displayed on the surface of the yeast Saccharomyces cerevisiae. Two reasons made us choose JFH1. First, JFH1 is a prototype 2a virus, which genetically belongs to the same genotype as those viruses identified in our chronically infected patients. Secondly, JFH1 is the best characterized strain and has been serving as the benchmark 2a strain for studies of anti-HCV antibody response. The four patients studied here were infected with HCV genotype 2a viruses based on analysis of full-length E1E2 sequences from the patients’ serum samples (Table 1). The specific steps and procedure for constructing the library have been described in greater detail previously (Zuo et al., 2011). To verify the quality of the constructed library and the specificity of the selection process, we first measured the size and coverage of library fragments along the JFH1 E1E2 envelope. The theoretical library size required to cover the full-length JFH1 E1E2 with ~100–600 bp fragments was estimated as 2 × [(X−100 + 1) + (X−101 + 1) + (X−102 + 1) + ... + (X−600 + 1)] where X is the number of nucleotides of the full-length JFH1 E1E2 envelope gene. As the JFH1 E1E2 envelope gene was 1677 bases, the calculated library size was about 1.34 × 10^6. Through serial dilution and counting the recombinant yeast colonies, the estimated size of the JFH1 E1E2 library was in fact well above 1.00 × 10^7 and was therefore sufficient to meet the theoretical requirements. To study the coverage along JFH1 E1E2, we randomly selected 132 clones from the library and conducted sequencing and sequence analysis. As shown in Fig. S1 (available in the online Supplementary Material), the fragments were able to cover the entire JFH1 E1E2 from the N to the C terminus. Further breakdown of selected fragments according to their size revealed that the 100–600 bp fragments constituted approximately 85 % of the library, consistent with our initial design. The wide range of fragment length, in particular those longer than 300 bp or 100 residues equivalent, would be expected to contain both linear and conformational epitopes (Lin et al., 2009).

To further confirm that the library indeed contained both linear and conformational epitopes, we used six mAbs (clones 3/11, 6/1a, 9/75, AR2A, AR2B and AR3C) with known epitope specificity to the HCV E1E2 glycoprotein to
select the JFH1 E1E2 combinatorial library through FACS. The first three (3/11, 6/1a and 9/75) recognized the linear epitopes, whilst the last three (AR2A, AR2B and AR3C) recognized the conformational epitopes (Hsu et al., 2003; Kong et al., 2013). Among the first three, two (3/11 and 9/75) were able to select out positive yeast clones, whilst one (6/1a) failed. Sequencing of these yeast clones revealed that the selected fragment sequences were variable in length but overlapped and clustered together in two distinct and separate locations along JFH1 E1E2 (Fig. 1a). Further analysis of common sequences shared by each of the clusters revealed two discrete stretches of amino acid residues: NTNGSWHINRTALNCNDSLN (aa 415–434) for mAb 3/11 and PTYTWGENET (aa 527–536) for mAb 9/75 (Fig. 1b). These sequences were virtually identical to the previously characterized epitopes of mAbs 3/11 and 9/75 (Flint et al., 1999; Hsu et al., 2003). In addition, the failure of mAb 6/1a to select positive yeast clones from the JFH1 E1E2 library was due to extensive residue substitutions in the epitope: five substitutions within the epitope of 10 residues, rendering it unrecognizable by mAb 6/1a (Flint et al., 1999; Hsu et al., 2003). Furthermore, for the last three mAbs (AR2A, AR2B and AR3C) recognizing the conformational epitopes, the selected fragments were relatively long (>250 residues) and contained the corresponding conformational epitopes for all three mAbs, suggesting that they must have been folded and displayed properly on the surface of the yeast (Fig. 1a). Sequencing analysis demonstrated that the selected fragments were largely located between residues 400 and 700 along the JFH1 E1E2 (Fig. 1a), which covered the entire E2 core (residues 412–645) used to solve the crystal structure of AR3C–E2 core complex (Kong et al., 2013). The significant overlap in fragment sequences with known epitopes observed here again reinforced the notion that the combinatorial library contained both linear and conformational epitopes and was well suited for studying the complex antibody recognition.

### Comprehensive profiling of the polyclonal antibody response against JFH1 E1E2 in chronically infected patients

In the current study, four separate serum samples (141, 192, 193 and 206) with the highest titres of neutralization activity were selected from five HCV chronically infected patients (Table S1) and analysed on their ability to select antigenic fragments from the combinatorial JFH1 E1E2 library. A total of 54, 42, 134 and 75 positive yeast clones were selected and sequenced from the 141, 192, 193 and 206 samples, respectively. Fig. 2(a) demonstrates the overlapping fragment sequences obtained from the positive yeast clones and their positions along the JFH1 E1E2 sequence. These fragment sequences were found to cover the entire JFH1 E1E2 sequence but with clear variations among the four samples and a bias towards certain regions within each. Overall, there were more fragment sequences identified in the E2 than in the E1 region, suggesting that more polyclonal antibody responses were generated against...
Fig. 1. Evaluation of the robustness and specificity of the combinatorial library of the JFH1 E1E2 glycoprotein and the selection process using mAbs with known epitope specificity. (a) Overlapping sequences of the positive yeast clones selected by mAbs 3/11, 9/75, AR2A, AR3B and AR3C were aligned with the original full-length JFH1 E1E2 sequence used for construction of the combinatorial yeast library. (b) Number of amino acid residues among the selected fragments along their corresponding positions in the JFH1 E1E2 glycoprotein. Red, dark blue, orange, purple and light blue bars represent the epitopes identified through the library selection process and their locations in relation to the JFH1 E1E2 glycoprotein. Below each bar, the actual linear epitope sequences, which closely resemble those identified previously (Flint et al., 1999; Hsu et al., 2003), are indicated. The transmembrane domains (TMD) for E1 and E2 as well as hypervariable regions (HVR1, HVR3, HVR2 and igVR) are also indicated.
Residue positions along JFH1 E1E2

Fragments selected by patients’ serum

Number of residues among the selected fragments

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the E2 region in these patients. This finding was in complete agreement with previous reports in which E2 was found to be more antigenic than E1 in infected patients (Edwards et al., 2012; Neumann-Haefelin & Thimme, 2013; Prince et al., 1999; Shimizu et al., 1994; Wahid & Dubuisson, 2013; Wang et al., 2011). Of note, the size of the selected fragments varied from 27 to 289 residues but were mostly between 50 and 150 residues, consistent with our initial design of the library to contain both linear and conformational epitopes (Zuo et al., 2011).

To characterize the polyclonal antibody response against E1E2 in a more quantitative manner, the frequency of each amino acid residue among all the fragment sequences was analysed and plotted along the JFH1 E1E2 sequence (Fig. 2b, shaded area). Similar to the biased selection of more E2 than E1 fragments (Fig. 2a), the number of amino acids recognized by the serum was greater in the E2 than in the E1 region, as reflected by the area under the curve (AUC) (Fig. 2b, shaded area). Whilst variability existed among different individuals it was estimated that, on average,
87.5% (37.1 + 7.7 + 39.5 + 3.2%) of the AUC corresponded to E2 whilst the remaining 12.5% to E1 (Fig. 2b, bottom panel). In other words, E2 was more than 7.0-fold more recognizable by the serum than E1. Such biased recognition was unlikely to be the result of E2 merely being longer than E1, as the ratio of the AUC for each sample was significantly different from the statistically expected value of 1.8 (280 vs 160 residues).

We went further to break down the shaded AUC into a total of five discrete major antigenic domains (D1–D5) through our previously described computer algorithm for sequence scanning and clustering (Zuo et al., 2011). The coloured peaks in Fig. 2(b) represented these domains, which were clearly variable in number and size among the different individuals, suggesting that different individuals mount different antibody responses against different regions of JFH1 E1E2. In patient 141, for example, five antigenic domains were identified each representing a sizeable fraction of the total polyclonal antibody recognition: D1, 19.0%; D2, 14.4%; D3, 18.7%; D4, 40.9%; and D5, 7.1% (Fig. 2b). In contrast, in patients 192, 193 and 206, the polyclonal antibody responses were targeted mostly to a single domain: D2 in patients 192 (52.7%) and 206 (76.4%), and D4 in patient 193 (84.9%) (Fig. 2b). Furthermore, analysis of selected fragments from each patient as well as combined fragments from all four patients revealed the average locations and proportions of each antigenic domain in total recognition by the polyclonal antibody in vivo (Fig. 2b; bottom panel, and Fig. 3a). D1 (aa 284–324) was estimated to be located within E1, whilst D2 (aa 407–428), D3 (aa 513–550), D4 (aa 637–678) and D5 (aa 690–720) were within E2 (Fig. 2b, bottom panel, and Fig. 3a). Among the five domains, it was apparent that D2 and D4 were the most antigenic, constituting roughly 76.6% (37.1% + 39.5%) of the total antibody recognition. Antibody responses against D1, D3 and D5, however, were relatively small, representing only 13.0%, 7.7% and 3.2% of the total recognition, respectively (Fig. 2b, bottom panel). Serum recognition of these antigenic domains was further confirmed by ELISA using 22 15-mer peptides across JFH1 E1E2 (Figs 3a and S2). These peptides were selected to be as close as possible to those autologous sequences identified in the four study subjects (93.9% for patient 141, 92.7% for patient 191, 93.6% for patient 193 and 92.7% for patient 206).

Interestingly, many previously identified mAbs from animals and humans also recognize epitopes within these antigenic domains (Edwards et al., 2012; Wahid & Dubuisson, 2013; Wang et al., 2011). For example, two human mAbs (clones IGH505 and IGH526) with broad neutralization activity against diverse HCV strains recognize a linear epitope (aa 312–327) overlapping D1 (aa 284–324) (Meunier et al., 2008). A group of neutralizing mAbs (clones AP33, 3/11, 95–2, H, Fab, AR3 and the CBH series) recognizing the conformational epitopes depended on the critical residues within D3 (aa 513–550) (Fig. 3b) (Edwards et al., 2012; Wahid & Dubuisson, 2013; Wang et al., 2011). D4 contained the various linear epitopes recognized by the AP or ALP series of mAbs (Fig. 3b) (Edwards et al., 2012; Wahid & Dubuisson, 2013; Wang et al., 2011). However, no mAb has so far been reported and mapped to residues within D5, which was close to the transmembrane domain of E2 (Fig. 3b).

Selective depletion of neutralizing activities by yeast clones displaying different antigenic domains

To evaluate further the contribution of major antigenic domains to overall recognition by neutralizing antibodies, we pooled the yeast clones expressing the HCV antigens corresponding to D1 in E1, D2–D3 in the N-terminal region of E2 (E2N) and D4–D5 in the C-terminal region of E2 (E2C) to specifically deplete antibodies from the four serum samples through repeated cycles of incubation and removal of antigen–antibody complexes. Serum neutralizing activities before and after depletion were compared against HCV pseudovirus bearing JFH1 E1E2 (Fig. 4). The most consistent and unexpected result from the four independent serum samples was that yeast clones displaying antigens containing D1 could selectively deplete a substantial proportion of the serum neutralizing activities (Fig. 4). The reduction in neutralizing activities measured as IC50 was a mean of 65% and could be as high as 74% in the case of patient 141 (Fig. 4). However, yeast clones displaying antigens containing D2–D3 (E2N) or D4–D5 (E2C) were only able to deplete a relatively smaller proportion of the neutralizing activities from a mean of 7% for the former and 30% for the latter (Fig. 4). These results suggested that, despite the majority of serum binding antibody being against E2, a large majority of them was probably limited in neutralizing activities. E1, on the other hand, contained more neutralizing epitopes than identified previously.

Fig. 4. Analysis of neutralization activity against each antigenic domain through serum absorption experiments. Comparison of serum neutralization before and after absorption with yeast clones expressing HCV fragments containing D1, D2–D3 (E2N) or D4–D5 (E2C). Left panels show neutralization activity against pseudotyped virus bearing the full-length JFH1 E1E2 glycoprotein through a series of dilutions, whilst right panels show the reduction in neutralization activity (IC50) after absorption with the respective yeast clones. CD20 represents a yeast clone expressing the irrelevant antigen CD20 used as a negative control during the absorption process.
DISCUSSION

We have reported here the comprehensive profiling of antibody responses against HCV in humans based on our previously developed robust technique of a combinatorial antigen library displayed on the surface of the yeast S. cerevisiae. Our results showed that there were significant differences in the breadth and magnitude of antibody responses against E1 and E2 glycoprotein among different subjects. In general, the binding response was targeted mainly against the E2 glycoprotein, whilst that against E1 was relatively less. Through a previously described computer algorithm for sequence scanning and clustering, we were able to further break down E1 and E2 into five major antigenic domains (D1–D5) preferably recognized by the polyclonal serum. In fact, a significant proportion of the antibody response against E2 was confined to D2 and D4, which contained the critical residues for receptor CD81 binding and for recognition of both neutralizing and non-neutralizing mAbs reported previously (Edwards et al., 2012; Wahid & Dubuisson, 2013; Wang et al., 2011). To our surprise, however, serum absorption with the yeast clones containing D1 resulted in a substantial reduction in serum neutralization activity, indicating that E1 contained more neutralizing epitopes than identified previously. It should be noted that there were only four patients studied here. The next logical step would be to apply the yeast library technique to a large cohort of HCV-infected individuals with distinct clinical, virological and immunological features. In this way, firmer conclusions could be drawn on the antigenic domains and their relevance to disease progression and immune protection.

The technique and quantitative data collected here are, to the best of our knowledge, the first of this kind for the study of the polyclonal antibody response against HCV in vivo. From the technique point of view, a yeast-based library offers several advantages over existing approaches. However, this does not mean that our approach is without shortcomings. It is to be expected that some of the conformational epitopes, in particular those formed through intermolecular interaction, would be lost during the fragmenting process for library construction. We have to bear this in mind when we analyse the data and draw conclusions from it.

Several intriguing findings emerged in the current study. First, whilst dominant antibody responses towards certain antigenic domains were shared, clear differences existed in the breadth and magnitude of responses among the four subjects. It is possible that different strains of HCV in different individuals could induce different antibody responses. In this regard, it needs to be pointed out that, although the four study subjects were infected by HCV genotype 2a, the combinatorial antigen library was built upon the 2a reference strain JFH1 E1E2, which was on average 11.2 % different from the autologous envelope glycoprotein sequences in these patients (Table 1). Future studies using the autologous envelope glycoprotein in the library construction will help to better understand the dynamics of antibody response against self viruses. Secondly, we were puzzled by the relatively smaller contribution of the E2-specific polyclonal antibody response to the overall neutralization activity, despite its dominance in binding activities. In particular, the dominant D2 recognized by the polyclonal antibody response overlapped with epitopes of several broad neutralizing mAbs such as AP33, 3/11, 95-2, H77.39 and HCV1 (Edwards et al., 2012; Wahid & Dubuisson, 2013; Wang et al., 2011). Thirdly, we were equally puzzled by the relatively large contribution of the E1-specific polyclonal antibody response to the overall neutralization activity, despite its small proportion in overall binding. Indeed, several E1-specific mAbs were isolated, some of which were cross-reactive to multiple genotypes whilst others had broad neutralization activity against multiple genotypes (Edwards et al., 2012; Meunier et al., 2011; Wahid & Dubuisson, 2013; Wang et al., 2011). In particular, vaccine strategies including E1 have shown their ability and superiority in inducing a broad neutralizing antibody response as well as protection efficacy compared with those without E1 (Garrone et al., 2011; Law et al., 2013; Meunier et al., 2011). Due to our limited knowledge of E1E2 structure and their interaction with corresponding host factors, we were uncertain about the mechanisms by which these E1-specific antibodies work. It is possible that they directly disrupt the interactions between HCV and receptors required for entry or fusion. Alternatively, they could interfere indirectly with the entry or fusion process through other unknown mechanisms. In either case, identification of E1 as a major target for neutralization antibodies provides an additional reference for a better understanding of the antibody response in vivo against HCV infection and should assist in the design of HCV vaccines aimed at enhancing and broadening the neutralization potential.

METHODS

mAbs, serum samples and HCV sequences. Six previously characterized HCV mAbs (3/11, 6/1a, 9/75, AR2A, AR2B and AR3C) with known epitope information (Flint et al., 1999; Hsu et al., 2003; Kong et al., 2013) were kindly provided by Dr Jane A. McKeating at the University of Birmingham, UK, and by Dr Mansun Law at the Scripps Research Institute, La Jolla, USA. Serum samples were collected from five HCV chronically infected patients with informed consent, and four (141, 191, 193 and 206) of these were selected based on their higher neutralization titres compared with the rest (Table S1). The study was approved by the institution’s ethics committee at You’an Hospital, Beijing, China. The clinical, virological and immunological characteristics of these subjects are summarized in Table 1. At the time of blood sampling, all patients were treatment naïve and their aspartate aminotransferase and alanine aminotransferase levels fell within the normal range (Table 1). HCV viral load in the serum was measured using the Roche COBAS TaqMan 2.0 System. To characterize the genetic features of HCV in the four patients, we isolated and purified viral RNA from the serum samples using an RNeasy Mini kit (Qiagen) and reverse transcribed them into cDNA using a One-Step RNA PCR kit (TaKaRa). HCV full-length envelope genes were amplified by nested PCR using the following primer pairs: 5’-GAAAGGCC- TTGTGCTACCTG-3’ and 5’-CCCTTGATGTACCAAGCAGC-3’ in the
first round, 5'-GATAGGGTGGCTGGAGGTG-3' and 5'-GCAAGA-
CGACCAGCTTTC-3' in the second round, at the same conditions of
94 °C for 2 min, followed by 30 cycles of 94 °C for 15 s, 60 °C for 30 s
and 68 °C for 4 min, with a final extension of 68 °C for 10 min. PCR
products were purified using a QIAquick Gel Extraction kit (Qiagen) and
ligated into the pcDNA3.3 TOPO vector (Invitrogen) for transformation
and sequencing. HCV full-length envelope sequences and their genetic
distances relative to that of JFH1 were analysed using MEGA4 and the
Biological Sequence Alignment Editor (Tamura et al., 2007).

Production of HCV pseudotyped virus and neutralization assay.
HCV pseudotyped virus was generated by co-transfection of the JFH1
full-length envelope-expressing plasmid together with the backbone
construct pNL43R-E-luciferase into 293 cells as described previously
(Hsu et al., 2003). At 48 h post-transfection, culture supernatant was
harvested, clarified, filtered (0.45 μm pore size; Millipore) and tested
for luciferase activity to standardize the viral input in the subsequent
neutralization analysis. A neutralization assay was conducted as
described previously (Hsu et al., 2003). In brief, 100 TCID50
pseudotyped virus was incubated with serially diluted patients’ serum
or mAbs in a 96-well plate in triplicate for 1 h at 37 °C. Approximately 1 × 10^5 Huh7.5.1 cells in 100 μl were then added to
each well and maintained for an additional 48 h at 37 °C. Neutralizing activity was measured by the reduction in luciferase activity compared with controls using the following formula:
neutralizing activity (%) = [1−(SC−CC)/(VC−CC)] × 100, where
SC, VC and CC are the luminescence of the samples, pseudovirus positive control and negative control, respectively. The IC50 and neutralizing curves were calculated based on a standard non-linear regression algorithm (log [inhibitor] vs normalized response) using GraphPad prism 5.0 software.

Construction and expression of JFH1 E1E2 combinatorial libraries
on the surface of the yeast S. cerevisiae. The construction of a
yeast library displaying the combinatorial HCV antigens was conducted as described previously (Zuo et al., 2011). In brief, the full-length HCV JFH1 envelope gene was amplified by PCR, purified and digested by DNase I into fragments about 50 bp. The digested fragments were reassembled to approximately 100–600 bp fragments through a controlled number of PCR cycles, which were then A-tailed and ligated to the modified yeast surface-displayed vector pCTCON2-T. The ligation products were transformed into Escherichia coli competent cells, amplified, extracted and then further transformed into the competent yeast cell line EBY100 using electroporation. The transformed yeast cells were partially spread on self-made SDCAA Amp plates and incubated overnight at 30 °C to estimate the number and insert sequences of colonies for quality control and to estimate the size of the library. The conditions for yeast growth and the induction of surface antigen expression in solution have been described previously (Zuo et al., 2011). In short, S. cerevisiae strain EBY100 yeast was first grown in SDCAA medium at 30 °C for 48 h. At the exponential growth phase, the yeasts were transferred to SGCAA medium for induction of antigen expression at 20 °C for 48 h before incubating with either serum samples or mAbs for subsequent analysis (Zuo et al., 2011).

Immunofluorescence staining and sorting of serum- or mAb-reactive yeast clones by FACS. The entire procedure was conducted as described previously (Zuo et al., 2011). Induced yeast cells (10^5–10^6) were collected by centrifugation (6000 rpm, 1 min; Eppendorf, 24-place fixed-angle rotor), washed twice with cold PBS and incubated with either patient serum (1:20 dilution) or mAb on ice for 1 h with occasional agitation. After washing three times with cold PBS, the cells were incubated with phycoerythrin-labelled anti-rat or anti-human IgG secondary antibody (1:200 dilution) on ice for another 45 min, washed again with PBS five times and analysed by FACS using Aria II (BD).

Identification of antigenic domains through sequence scanning and clustering. Antigenic domains were identified based on the overlapping fragment sequences through a computer algorithm described previously (Zuo et al., 2011). In brief, the obtained fragment sequences were first translated into amino acid sequences and aligned against the full-length JFH1 E1E2 amino acid sequence. A sliding window of 10 aa was used to scan across the entire alignment from the N to the C terminus one residue at a time, although the size of the window and scanning steps could be adjusted. When a window found fragment sequences containing at least 5 aa or ≥50 % identical to those in the full-length sequence, the position of the given window was scored based on the number of fragment sequences identified. The window containing the highest number of fragment sequences was classified as the first antigenic domain, which was then plotted based on the frequency of amino acid residues along their corresponding positions in the full-length HCV sequence. The scanning process continued again from the beginning until all the fragment sequences had been assigned to appropriate antigenic domains. For clarity, all the antigenic domains were colour coded and have been kept consistent in all of the figures.

Absorption of antigen-specific antibodies through serum-reactive yeast clones. To evaluate the contribution of major antigenic domain-specific antibodies to overall neutralization activity, serum samples were incubated with a mixture of yeast clones expressing HCV antigens containing D1 (E1), the D2–D3 (E2N) or D4–D5 (E2C) to specifically absorb or remove antibodies targeting these regions. The selected yeast clones were induced independently in SGCAA medium, mixed with equal proportion to make approximately 2–3 × 10^6 cells, and incubated with 500 μl serum on a rotary shaker at 4 °C for about 3 h. The yeast cells and antibody complex were removed by centrifugation. The same procedure was repeated five times by adding fresh yeast mixture during each cycle of absorption until no antibody response was detectable against the corresponding yeast mixture measured by FACS. The resultant serum samples were then filtered (0.22 μm diameter filter; Corning), evaluated for their neutralizing activity and compared with that of untreated serum samples.

Measurement of JFH1 E1E2-specific binding antibody in the serum by peptide ELISA. To compare the serum profile obtained through the yeast surface-display system, serum binding antibody against JFH1 E1E2 was also analysed by peptide ELISA. A total of 22 15mer peptides were selected and synthesized along the JFH1 E1E2 sequence (ChinaPeptides Co.) to be as close as possible to those autologous sequences identified in the four study subjects (93.9 % for patient 141, 92.7 % for patient 191, 93.6 % for patient 193 and 92.7 % for patient 206). These peptides were coated on a 96-well plate in coating buffer (150 mM Na2CO3, 350 mM NaHCO3, pH 9.6) at 4 °C overnight. Plates were washed with PBST (0.1 % Tween 20 in PBS) five times and blocked with 100 μl of 10 % goat serum at 37 °C for 2 h. After extensive washing with PBST, heat-inactivated serum samples (100 μl, 1:1400 dilution in coating buffer) were added to each well and incubated for 1 h at 37 °C. After washing with PBST, the antigen–antibody complexes were incubated with HRP-conjugated anti-human IgG secondary antibody (1:2000 diluted in PBST; Promega), washed again to remove unbound secondary antibody and reacted with the HRP substrate 3,3',5,5'-tetramethylbenzidine (Beyotime). The reaction was then terminated with 50 μl 2 M H2SO4 and quantified by recording the absorbance value at 450 nm with an ELISA Reader (model 680; Bio-Rad).

Statistics. The correlation between each antigenic domain presented as either AUC or absorbance with neutralization activity (IC50) was considered as statistically significant when P<0.05.
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