Identification of a novel epitope in the C terminus of hepatitis C virus-E2 protein that induces potent and cross-reactive neutralizing antibodies

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Abstract
Hepatitis C virus (HCV) is a leading cause of chronic viral hepatitis, but an effective vaccine is still not available to prevent infection. Use of neutralizing antibodies could be a potential therapeutic option. In this study, the presence of anti-HCV antibodies in HCV-infected patients was assessed from 50 patients and the presence of neutralizing antibodies was examined using ‘hepatitis C virus-like particles’. Antibodies from two samples exhibited significant inhibitory activity, suggesting that these may neutralize viral infection. Antigenic determinants generating the neutralizing antibodies from these two samples were delineated by epitope mapping using the core, E1 and E2 regions and a stretch of 45 amino acid peptide (E2C45) derived from the C-terminal region of HCV-E2 protein (aa 634–679) was designed. Results suggest that this hitherto uncharacterized region has the potential to generate neutralizing antibodies against HCV and thus be effective in preventing virus entry into liver cells. Computational analysis of the structure of the modelled peptide (E2C45) suggested high conformational entropy for this region. Furthermore, E2C45 peptide-generated antibodies could block virus entry and monoclonal antibodies generated against this peptide could also significantly reduce virus replication in a cell culture system. It is possible that the inhibition could be partly due to a conformational alteration of the CD81-binding region, preventing virus attachment to liver cells. In conclusion, this work focused on the discovery of a novel epitope at the C terminus of E2 that induces potent neutralizing antibodies in HCV-infected patients.

INTRODUCTION
Hepatitis C virus (HCV) infection is a major health problem worldwide, affecting more than 170 million people, and causing viral hepatitis, which often leads to hepatocellular carcinoma [1, 2]. In India, HCV genotype 3a is prevalent followed by genotype 1b [3].

HCV is a small-enveloped, single-stranded RNA virus [4]. The envelope glycoproteins E1 and E2 are structural proteins located on the surface of HCV and responsible for binding to cells and entry. They are N-linked glycosylated proteins with an N-terminal ectodomain and a C-terminal hydrophobic membrane anchor. The major neutralizing determinants are present on the envelope proteins E1 and E2 [5].

There is evidence of interactions of numerous host proteins that are reported to be involved in viral entry. These include glycosaminoglycans (GAGs) [6, 7], LDL receptor (LDLR) [8, 9], four scavenger receptor class B type I (SR-BI) [10, 11], tetraspanin CD81 [11], and tight junction proteins claudin-1 (CLDN1) [12] and occludin (OCLN) [13]. In addition to these receptors, there are also several entry factors including epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2) [14], transferrin receptor 1 (TfR1) [15] and cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) [16]. However, it was previously shown...
that these receptors individually are not sufficient to allow viral entry into hepatocytes [17, 18]. Neutralizing epitopes have also been described that are different from the CD81 binding region [4]. The recently reported crystal structure of E2 has confirmed that a broadly conserved neutralizing epitopic stretch exists around the discontinuous CD81 binding site encompassing residues 412–424, 436–442 and 520–535 that harbour contact residues for many neutralizing antibodies [18].

At present, no vaccine is available to prevent HCV infection, and PEGylated IFN-α-based standard anti-virus treatment is less efficacious against some of the prevalent genotypes of HCV. The lack of proof-reading activity of the RdRp results in the production of different virus variants and thus generation of effective anti-HCV therapeutics is a major challenge. However, the efficacy of HCV treatment has improved through the design of multiple direct-acting antivirals (DAAs) which include protease inhibitors, and NS5A and NS5B inhibitors. The protease inhibitors generally inhibit the activity of NS3 either by blocking the catalytic site or by inhibiting the NS3–NS4A interaction. The first-generation protease inhibitors Telaprevir and Boceprevir are used in combination with PEG-IFN + Ribavirin. Both these inhibitors enhanced the sustained virologic response by up to 70 % in HCV patients infected with genotype 1 [19, 20]. Additionally, several other inhibitors, such as Simeprevir, Ledipasvir, Daclatasvir and Sofosbuvir, have been approved recently by the Federal Drug Administration. However, these drugs are associated with side effects and their efficacy against different genotypes is limited [21]. Thus, there is a need for the development of an effective vaccine and new treatment regimens. Virus neutralizing antibodies (nAbs) are potential therapeutics for prevention/management of viral infections [22] and could be explored for better alternative strategies. E2-specific antibodies derived from human sera [23, 24], polyclonal IgG derived from E1–E2 immunized mice [25] and HCV envelope glycoprotein-specific monoclonal antibodies (mAbs) [26–28] have been reported to neutralize HCVcc (HCV cell culture) in vitro. These nAbs effectively block interactions with receptors or inhibit post-entry events such as viral uncoating and replication [29].

As mentioned above, virus nAbs are probable therapeutics for treating viral infections. They are also useful reagents for studying the antigenicity and function of viral surface proteins [30]. Several surrogate models of infection such as animal infection and cell and receptor binding assays have highlighted the potential role of antibodies in both acute and chronic infections. It is imperative to note that not all antibodies that inhibit binding of virus to cell receptors as observed in the in vitro assays necessarily neutralize infection. It has been shown that the majority of antibodies that demonstrate broad neutralization of infection and/or inhibition of receptor binding are directed against linear epitopes within E2 [31].

There are several reports on nAbs generated to the C-terminal regions of E2 protein. Giang et al. [32] isolated 73 human mAbs, recognizing five distinct antigenic regions, the majority of which were able to neutralize viral infection. In particular, mAbs recognizing discontinuous epitopes encompassed in the region 639–698 possess exceptionally broad neutralizing activity towards different genotypes of HCV [32].

In this study, using baculovirus-generated HCV-like particles (HCV-LPs) we isolated anti-viral antibodies from patients with acute or chronic HCV infection that were able to specifically inhibit the binding of HCV-LP to hepatic cells. With serum samples obtained from HCV-infected patients, we showed for the first time that neutralizing IgG antibodies generated to the C-terminal region of E2 protein (aa 634–679) were able to successfully reduce HCV genotype 3a infection. More importantly, a stretch of 45 aa (E2C45) encompassing the neutralizing epitopes was designed and mAbs raised against this peptide showed significant inhibition of HCV in a virus neutralization assay.

**RESULTS**

**Inhibition of HCV-LP binding to Huh7 cells by serum-derived anti-HCV human antibodies**

Serum samples were obtained from 50 HCV-infected patients and the presence of anti-HCV nAbs was tested. To inhibit binding of HCV-LPs to Huh7 cells, purified IgG obtained from HCV-infected patient serum (1 µg) was incubated with HCV-LPs of genotype (gt) 3a or 1b followed by addition to Huh7 cells. Binding of HCV-LPs to Huh7 cells was measured by flow cytometry. Of the 50 HCV-infected serum samples tested for the presence of nAbs (Fig. 1a), two samples (C002 and C007) inhibited the binding of the HCV-LPs to Huh7 cells (>50 %). Sample C002 exhibited ~71 % inhibition of binding of gt3a and 72 % inhibition of gt1b. Sample C007 showed 65 % inhibition with gt3a and 58 % with gt1b. None of the five control sera showed any inhibition (Fig. 1a). Interestingly, among 50 HCV-positive patients with acute symptomatic hepatitis C, in the two patients (C002 and C007) whose sera inhibited HCV-LP binding to Huh7 cells, there was subsequent resolution of HCV infection [as determined by ELISA and reverse transcriptase PCR (RT-PCR)]. As seen from Table 1, 19 of 50 patients were infected with gt 1 and 14 with gt 3. The binding profile of antibodies from patients C002 and C007 did not demonstrate genotype-specific inhibition (Fig. 1a).

**Inhibition of virus entry by human antibodies in HCV cell culture**

Flow cytometric analysis suggested that two of the patient sera, C002 and C007, inhibited HCV-LP binding to Huh7 cells. To verify whether this property is also shown when virions of hepatitis C are used, virus neutralization assays were carried out using HCV-JFH1 (gt2a) virus in the infectious cell culture system. The virus was pre-incubated with the purified IgG (20 µg ml⁻¹) from HCV-infected patient sera (C002 and C007) for 1 h at 37 °C before infection. As a negative control, HCV negative healthy serum (N23) was used. Three days post-infection, virus replication was
monitored by quantification of HCV negative strand RNA using real-time RT-PCR. IgG from C002 and C007 showed a trend similar to the inhibition of HCV-LP binding to cells. Huh7.5 cells infected with JFH1 virus in the presence of C002 antibody (2 µg) showed a nearly 84% reduction in intracellular HCV RNA level, while C007 showed a decrease of about 45% at the same concentration. The serum from an uninfected individual (N23) had no effect on inhibition, as expected, demonstrating the specificity of the assay (Fig. 1b).

Identification of epitopic regions on HCV-LP recognized by human antibodies

To delineate which structural protein is mostly responsible for generating nAbs, ELISA was performed using peptide pools representing gt3a core, E1 and E2 as antigens and IgG from C002, C007 and healthy individuals. The results (Fig. 2a) revealed highest reactivity with E2 peptide pool (384–752 aa), suggesting that E2 protein may contain the majority of the neutralizing epitopes. To narrow down the regions within E2 further, a peptide library (from NIH) that covered the entire E2 glycoprotein consisting of 16-mer peptides each with an overlap of 11 aa was scanned using sera from HCV-infected patients (C002, C007, HCV04 and HCV16). Analysis of pools of 10 consecutive peptide sub-pools (Fig. 2b) revealed that the IgG response for both C002 and C007 was most pronounced against the C-terminal part of the E2 glycoprotein (P4, P5 and P6) (Fig. 2c). Minor reactivity was detected to the other regions of the protein (pools P1, P2 and P3) (Fig. 2c). However, non-neutralizing anti-HCV IgG (HCV04 and HCV16) did not show a significant difference in reactivity with any particular region (Fig. 2d). These observations suggest that the C-terminal part of E2 was able to generate nAbs in HCV-infected humans. Among the two peptide sub-pools P5 and P6, pool P5 showed substantial reactivity with C002 and C007. ELISA was then carried out with individual peptides from pool P5 (634–705 aa) (Fig. 3a). A considerable reactivity of the peptides P5.1 (aa 634–649), P5.2 (aa 639–654), P5.5 (aa 655–672) and P5.6 (aa 662–679) with the C002 and C007 IgGs suggested that these regions could be involved in generating the nAbs present in the patients’ sera (Fig. 3b).

Based on the above data, a 45-mer peptide (E2C45) comprising sequences from regions P5.1, P5.2, P5.5 and P5.6 (Fig. 3c) was synthesized and tested for its ability to generate nAbs against HCV-LP.

Inhibition of HCV-LP binding to Huh7 cells by mouse anti-E2C45 peptide serum

Since the availability of human polyclonal anti-HCV serum was limited, it was necessary to generate polyclonal anti-E2 antibody in a small animal model such as mouse for further characterization. Thirty micrograms of BSA-conjugated E2C45 peptide was injected as primary immunization followed by 15 µg booster dosage. Significant anti-E2C45 and anti-HCV–LP antibodies were detected 52 days post-immunization (data not shown). A dose-dependent inhibition of binding of the HCV-LP gt3a as well as 1b to Huh7 cells by the mouse anti-E2C45 antibodies was seen (Fig. 4a, b). With 20-fold excess of purified IgG (~20 µg ml⁻¹), there was almost ~70% inhibition of HCV-LP gt3a binding to Huh7 cells. However, in the case of gt1b, it was lower (50%, Fig. 4b). Thus, it was observed that the peptide E2C45 generated nAbs that could inhibit virus binding to hepatic cells. These results suggest that antibodies generated against the peptide could prevent the interactions of HCV-LP with cells.

Anti-E2C45 peptide antibodies neutralize HCV-JFH1 virus replication in a cell culture system

High concentrations of nAbs in serum are usually directly correlated with virus clearance. To test the potential of the antibodies generated in neutralizing HCV, a neutralization assay using gt2a virus was performed. At the
increasing concentrations (10, 25 and 50 µg ml\(^{-1}\)) of antibody tested, there was a significant decrease of the viral RNA levels (Fig. 4c) and 50 µg ml\(^{-1}\) concentration showed almost 80 % reduction. Purified IgG from normal mouse serum and serum from mice immunized with an unrelated peptide were used as negative controls that did not show inhibition.

Inhibition of HCV-LP binding to Huh7 cells by mAbs against E2C45

Encouraged by the observation of inhibition of cellular HCV-LP binding with the mouse anti-E2C45 antibody, we next generated a panel of anti-E2C45 mAbs. BALB/c mice were immunized with the peptide E2C45 and hybridomas were established by fusion of splenocytes with mouse myeloma cells. Four mAbs, namely A8A11, C10E8, H3D12 and H9G4, were obtained against E2C45. It was observed by ELISA that all four mAbs recognized HCV-LP and the peptide E2C45 (data not shown). Finally, to determine the epitopic regions of the respective mAbs, ELISA was performed using the overlapping peptides (Fig. 5a). As evident from the results, mAbs A8A11, C10E8 and H3D12 recognized the peptides 5.5 (GERCDIEDRDRSEQHPLL) as well as 5.6 (DRDRSEQHPLLHSTTELA) lying between aa 654 and 678, whereas mAb H9G4 recognized peptide 5.5 only. Therefore, it is probable that the antigenic region able to generate anti-HCV nAbs lies between these two peptides (DRDRSEQHPLL) (aa 661–671). Furthermore, to characterize the two mAbs (A8A11 and C10E8) that showed maximum reactivity with E2C45 peptide as well as HCV-LP in ELISA, their reactivity to strain HCV-JFH1 in the cell culture system was tested by an immunofluorescence assay. As shown in Fig. 5(b), a combination of these two mAbs was able to detect the virus in infectious cell culture (Huh7.5 cells, detailed in Methods). To further characterize the mAbs, HCV-LPs as well as purified recombinant E1E2 proteins (batch I and batch II) were also specifically detected using A8A11 and C10E8 mAbs independently, as shown in Western blot analysis (Fig. 5c). Furthermore, to show the specificity of the two mAbs reacting with HCV-LP and rE1E2, HCV non-structural protein 3 protease domain (NS3\(^{pro}\)) was used as a non-specific protein control in the

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NA, Not applicable.

*Patient sera showing virus neutralization activity.
†Patient sera showing non-neutralizing activity and hence taken as negative control.
Western blot analysis. Since all of the mAbs exhibited considerable specificity in reactivity with virus-like particles (VLPs) as well as E2C45 peptide, the inhibition of binding of HCV-LPs to Huh7 cells by the mAbs was studied by flow cytometric analysis. mAbs C10E8 and A8A11 demonstrated potent neutralizing characteristics. C10E8 was able to inhibit the cellular HCV-LP binding significantly (~75%) with 10 µg of purified IgG. The inhibition by mAb A8A11 was lower than C10E8 (~70%), whereas the other two mAbs showed a much lower inhibitory effect (<50%) (Fig. 5d).

Inhibition of virus entry by the mAbs in HCV cell culture

Flow cytometric analysis suggested that the E2-specific mAbs A8A11 and C10E8 inhibited HCV-LP binding to Huh7 cells. To verify whether the antibodies can also inhibit the binding of virions of hepatitis C, neutralization assays were performed using JFH1 virus. Huh7.5 cells infected with JFH1 virus in the presence of the mAbs (A8A11 and C10E8) at 100 µg ml⁻¹ showed nearly 95% reduction in intracellular HCV RNA level (Fig. 5e), which was much more than the inhibition by individual mAbs at the same concentration.

Similarly, to test the neutralization ability of the two mAbs A8A11 and C10E8 for HCV-gt3, neutralization assays were performed with gt3 virus derived from a patient’s serum. To assess propagation of the serum-derived virus in Huh7.5 cells the HCV (gt3)-positive patient serum was used to infect Huh7.5 cells and the viral RNA was quantified by RT-PCR. A gradual increase in HCV RNA levels was observed up to 96 h, suggesting steady propagation of the serum-derived virus (Fig. S1, available in the online Supplementary Material). The virus was pre-incubated with the individual or combination of mAbs (50 and 100 µg ml⁻¹ each) at 37 °C for 1 h followed by incubation of the complex with Huh7.5 cells. The percentage of the negative strand RNA level was quantified by quantitative (q)RT-PCR at 72 h post-infection. With gt3 virus there was up to 60% reduction (Fig. 6a) in viral RNA level (negative strand) with the concentration tested in the previous experiment (Fig. 5b).

Inhibition of virus propagation using infectious foci by the mAbs

To further confirm the inhibition of virus titre by the mAbs, a foci reduction assay was performed. The results showed a progressive reduction in the number of infected foci with a gradual increase in the mAb concentration individually or in combination. A considerable decrease (81%) in the number of infectious foci was observed with mAb combinations at 100 µg ml⁻¹. Thus, the infectious foci reduction assay results (Fig. 6b, c) were consistent with the neutralization assay results (Figs 5e and 6a).

Structural analysis of HCV E2 glycoprotein

Two crystal structures for HCV E2 glycoprotein were available in the Protein Data Bank (PDB) (PDB codes: 4WEB and 4MEF) [33, 34]. This E2C45 region mapped to a 45-residue stretch near the C-terminal end of HCV E2 protein. But the missing coordinates for the C-terminal region of E2C45 in the crystal structures suggested the a flexible nature of this region. However, the first 18 residues in the E2C45 stretch was ordered as a β-hairpin and therefore coordinates of atoms in this region were available.

To understand the possible structural state of the E2C45 region, a template structure for the same was searched. However, because the E2C45 did not show significant similarity to a stretch in proteins of known three-
dimensional structures, it was decided to build a model for this region using a state-of-the-art development server especially for ab initio modelling, known as Robetta (http://robetta.bakerlab.org) [35]. To generate a model with high confidence, the entire E2 protein sequence was given as input to Robetta and we compared the resulting high-scoring model with the available crystal structure. A model for the entire E2 protein was generated because the structure of the C-terminal region, which is the region of interest for this study, may depend on the structure of the rest of the protein.

We were able to assess much of the model in comparison to the available crystal structure. TMalign (http://zhanglab.ccmb.med.umich.edu/TM-align) [36] was used to compare the modelled and crystal structures. The two structures superposed well (Fig. 7a) with a TM score of 0.959 and root-mean-square deviation of 1.11 Å, generating high confidence in the model. Interestingly the first 18 residues of the 45-residue C-terminal stretch visible in the crystal structure formed a β-hairpin structure, which is also perfectly modelled in the structure generated by Robetta (Fig. 7a).

Having gained confidence in the generated model, the spatial localization of the 27-residue stretch (after the first 18 residues of the 45-residue C-terminal stretch) was examined. As seen from Fig. 7(a) the 27-residue stretch was devoid of α-helical or β-sheet regions and was predicted to largely comprise a loop structure. This aspect was entirely consistent with the secondary structure prediction by PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred) [37], which predicts a loop structure for the entire 27-residue region (Fig. 7b). More importantly, solvent accessibility calculations showed that 40 of 45 residues in the C-terminal region were solvent-exposed, which was consistent with the expected exposure of this region to the antibody. To explore the potential of the E2C45 region to fold by itself, a structure for the E2C45 region alone (Fig. S2, available in the

Fig. 3. Mapping of individual peptides of E2 sub-pool P5 recognized by IgG from C002 and C007. (a) Schematic showing individual peptides corresponding to the sub-pool P5 of HCV envelope protein E2. (b) Reactivity in ELISA of IgG from C002 (light grey bar), C007 (dark grey bar) and healthy control (black bar), using peptides corresponding to E2 peptide sub-pool P5. The graphical data indicate mean±sd. *P<0.05, **P<0.005, ***P<0.0005. The assay was performed in triplicates and repeated at least three times. (c) Schematic diagram depicting the complete sequence of peptide E2C45 derived from the E2 protein.
The CD81 binding site (G_{524}^{527} Y_{528}^{529} N_{530}^{531} E_{534}^{535} D) [34] was designed. This peptide was conjugated to BSA and antiserum was raised in mice. The specificity of the polyclonal antibodies to the peptide spanning the 518–537 aa region of E2 protein, as well as complete HCV-LP, was confirmed by ELISA where the reactivity of the polyclonal antibodies was tested against 100ng of HCV-LP and 500ng of peptide spanning the 518–537 aa region of E2 protein (Fig. 7c). Surprisingly, antibodies against the peptide spanning the 518–537 aa region of E2 protein showed better reactivity with HCV-LP as compared to itself (Fig. 7c). To show whether conformation alteration of HCV-LP occurs upon binding of the mAbs, ELISA was performed using HCV-LP as coating antigen and binding of biotinylated polyclonal antibodies against the peptide spanning the 518–537 aa region of E2 protein to VLPs in the presence and absence of mAbs against E2C45 peptide. The results (Fig. 7d) showed that the presence of mAbs A8A11 and C10E8 seem to have caused an alteration in the conformation of the CD81-binding region, leading to inhibition of binding of the polyclonal antibodies against the peptide spanning the 518–537 aa region of E2 protein. In contrast, and importantly, binding of mAbs H3D12 and H9G4 did not decrease binding of the polyclonal antibody, indicating that their binding to HCV-LP did not alter the structural conformation of the CD81 binding region.

Furthermore, to directly assess the ability of these mAbs to inhibit the binding of the HCV-LP to receptor (CD81), we carried out an experiment in CHO cells that lack CD81. We used the CHO-CD81 cell line that constitutively expresses CD81 for this purpose [38]. The results demonstrated a modest inhibitory effect of the mAbs in the CHO cell line expressing CD81 compared to control. As seen in Fig. 7(e), both mAbs were able to considerably block (30 to 40%) the binding of HCV-LPs to CHO-CD81 cells as compared to a non-specific mAb E1B11 [38], reconfirming that the neutralizing effect of the mAbs could be due to a conformational change in the CD81 binding site indirectly.

**DISCUSSION**

Although a majority of HCV infections evolve to chronicity, around 25% of individuals clear the infection spontaneously. It is believed that the type and strength of the host immune responses during the acute phase of HCV determines the infection outcome [39]. However, controversial results have been obtained on the contribution of nAbs in early studies [40]. In contrast, earlier studies involving acutely infected patients suggested that there may not be a major role for nAbs in spontaneous control of infection [41]. However, other studies involving HCV outbreaks with a defined inoculum showed that nAbs were induced in the early phase of infection in patients who subsequently cleared the virus [42]. These data indicate that early production of nAbs may contribute to control of the virus during the acute phase of HCV infection and facilitate viral elimination by cellular immune responses. In our study, serum samples from

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**Fig. 4.** Inhibition of HCV-LP binding to Huh7 cells by anti-E2C45 antibody. Alexa 488-labelled HCV LPs were incubated with IgG from mice immunized with E2C45. The y-axis depicts percentage inhibition of binding of 3a HCV-LPs (a) or 1b HCV-LPs (b) to Huh7 cells; the x-axis represents different concentrations of purified antibody. (c) HCV-JFH1 virus was pre-incubated with normal mouse IgG (50 µg ml\(^{-1}\)) or different concentrations (50, 25 and 10 µg ml\(^{-1}\)) of mouse anti-E2C45 antibody. Three days post-infection, HCV RNA levels were quantified using real-time RT-PCR. Data were normalized with respect to GAPDH. A non-neutralizing antibody raised against 3a HCV-LP (E1B11) was used as a negative control. The error bar represents mean±SD from three biologically independent experiments performed in triplicate. Asterisks represent a statistically significant difference compared to the negative control (**P<0.0002).
HCV-infected patients were analysed for the presence of nAbs. As seen from the results, two of the 50 HCV-infected patients harboured antibodies that were able to neutralize the VLPs binding to target cells. More importantly, when they were tested with HCV-JFH1 virus, there was a significant reduction in the viral RNA, suggesting the presence of nAbs in the sera. When the patients’ histories were traced, interestingly it was found that these two patients (C002 and C007) were actually able to cure the HCV infection at the acute stage itself, thus providing a strong correlation between the presence of nAbs and clearance from virus infection.

The major target of nAbs is the virus E2 envelope glycoprotein. In the present work, a stretch of 45-mer amino acid on the E2 protein was identified that was capable of generating HCV-nAbs in immunized mice. When compared with other stage-specific serum samples from HCV-infected patients, it was observed that the occurrence of these nAbs was at a considerably higher level in C002 and C007 as compared to other samples (data not shown). The importance of the E2C45 region was further demonstrated by the observation that mAbs generated against this region were able to reduce viral RNA synthesis in a cell culture system (up to ~95%).

**Fig. 5.** Identification of epitopes on E2C45 recognized by E2C45-specific mAbs and neutralization of virus. (a) Graph showing the reactivity of the mAbs A8A11 (black bar), C10E8 (dark grey bar), H3D12 (light grey bar) and H9G4 (white bar) with 16-mer overlapping peptides of E2C45. P5.4 was used as a negative control and peptide E2C45 as a positive control. The graphical data were analysed using a two-way ANOVA test; ***P<0.0001. (b) Immunofluorescence assay to show the reactivity of the combination of mAbs A8A11 and C10E8 with HCV-JFH1 virus. The upper panel shows the reactivity of the combination of mAbs in HCV-JFH1-infected cells and the lower panel shows a negative control (without HCV-JFH1 infection). (c) Western blot analysis showing the reactivity of the mAbs A8A11 and C10E8 with purified HCV-LPs and purified recombinant E1E2 protein. HCV non-structural protein 3 protease domain, NS3prot, was used as a negative control. Lower panel shows the reactivity of NS3prot with anti-NS3prot antibody. (d) Alexa488-labelled gt3a HCV-LPs were incubated with mAbs (5 µg ml⁻¹ represented by white bar and 10 µg ml⁻¹ represented by black bar) and inhibition of binding to Huh7 cells was measured by flow cytometry. The x-axis depicts the different mAbs and the y-axis shows the percentage inhibition of binding of HCV-LPs to Huh7 cells. (e) HCV-JFH1 virus was pre-incubated with mAbs C10E8 and A8A11 (50 and 100 µg ml⁻¹) or a combination of the mAbs before infecting Huh7.5 cells. Three days post-infection, HCV negative strand RNA was measured using real-time RT-PCR. GAPDH was used as an internal control. The graph shows the relative decrease in HCV RNA levels in the presence of different concentrations of mAbs. A combination of two unrelated antibodies (100 µg ml⁻¹) was used as a negative control. The error bar represents mean±SD from three biologically independent experiments performed in triplicate. Asterisks indicate statistically significant differences compared to the negative control (***P<0.0001; **P<0.0002).
Fig. 6. Inhibition of binding of serum-derived HCV-gt3 virus to Huh7.5 cells using E2C45 mAbs. (a) HCV-infected patients’ serum-derived virus was pre-incubated with two different concentrations (50 and 100 µg ml\(^{-1}\)) of anti-E2C45 mAbs C10E8 and A8A11 individually or in combination and added to Huh7.5 cells. Three days post-infection, HCV RNA levels were quantified using real-time RT-PCR. Data were normalized with respect to GAPDH (an endogenous control). A combination of two non-specific mAbs was taken as a negative control. The data indicate mean±SD. *P<0.05, **P<0.005. (b) Infectious foci assay. JFH1 virus was incubated with different concentrations of the individual mAbs or their combinations (50 and 100 µg ml\(^{-1}\)) and added to Huh7.5 cells. After 3 days, cells were incubated with HCV anti-NS5B antibody followed by Alexa Fluor 488-conjugated anti-mouse IgG antibody. The infected foci produced were quantified using a confocal microscope. (c) The percentage reduction of infectious foci from the neutralized and non-neutralized samples were quantified and plotted. Asterisks represent statistically significant differences compared to the negative control (**P<0.0002).
E2 protein were found within HVR1 [43]. However, the neutralizing epitope identified in our study is at the C-terminal region. It is possible that binding of this nAb to virus is likely to result in the change in conformation of the CD81-binding region, which precludes interaction of the virus with receptors. Moreover, there were some interesting results observed in the virus neutralization in the presence of E2C45 mAbs. Our results showed that when equimolar concentrations of all four mAbs (A8A11, C10E8, H3D12 and H9G4) were used for HCV-LP inhibition assay in combination, there was neutralization activity up to 75%. However, when used individually, mAbs A8A11 and C10E8 showed much better inhibition compared to the combination but mAbs H3D12 and H9G4 did not show appreciable inhibition, suggesting that these two latter mAbs are not involved in neutralization and probably might interfere with overall effectiveness if used in combination with other mAbs. This result is in agreement with the concept that the presence of non-neutralizing IgGs could cause partial blockage in the effective neutralization activity [44].

From the structural studies, it must be pointed out that the presence of loops in the epitopic region in the C terminus suggests the highly flexible/dynamic nature of this region.

Fig. 7. Change in conformation of the CD81 binding site upon binding of mAbs. (a) The structure of HCV E2 protein predicted by Robetta was superposed on the crystal structure. Blue shows the crystal structure of HCV E2 glycoprotein (4mef); green shows the structure predicted by Robetta; red shows E2C45 with P5.5 and P5.6 region in orange and region 663–668 in yellow; magenta shows the CD81 binding region. (b) Predicted secondary structure by PSIPRED for E2C45 where H is helix, E is strand and C is coil. (c) Characterization of polyclonal antibodies against the peptide spanning the 518–537 aa region of E2 protein. The reactivity of the polyclonal antibodies against the peptide spanning the 518–537 aa region of E2 protein was analysed by ELISA using HCV-LP (circles and lines) as well as the peptide spanning the 518–537 aa region of E2 protein (squares and lines) as antigen. (d) Alteration in CD81 binding region in the presence of bound anti-E2-C45-specific mAbs to VLPs. The mAbs A8A11, C10E8, H3D12 and H9G4 were added to immobilized HCV-LP followed by the addition of biotinylated antibodies against the peptide spanning the 518–537 aa region of E2 protein raised in mouse. The y-axis shows the percentage inhibition of binding of antibody to HCV-LP in the presence of the individual mAbs. The error bars represent SEM. **P<0.005. The assay was performed in duplicate and repeated at least three times. (e) Inhibition of HCV-LP binding to CHO-CD81 cells by mAbs. The mAbs (A8A11 and C10E8) were incubated with labelled HCV-LPs and then added to CHO-CD81 cells and binding of HCV-LPs was measured by flow cytometry. Normal mouse IgG (NMIgG) and a non-neutralizing antibody (E1B11) were used as negative controls and E3D8 (epitope lying on the CD81 binding site) was used as a positive control. Asterisks represent statistically significant differences compared to the negative controls. The y-axis depicts the percentage inhibition of HCV-LP binding to Huh-7 cells. All data are pooled from three independent experiments performed in duplicate. The graphical data were analysed using a t-test (*P<0.0136, **P<0.0034).
and therefore most of this region is expected to be solvent-exposed. Our model reinforces this expectation and this region is likely to be ordered better with binding of the antibody. In the absence of an antibody, however, this region might have multiple conformational states (with one of them shown in the model generated by Robetta). All the conformational states are likely to be exposed, with the conformational entropy reduced enormously upon binding to the antibody.

In fact, other than HVR1, several other neutralizing epitopes have been identified in E2 with the help of mAbs [22]. Interestingly, the majority of these antibodies target epitopes located in Domain I, and they neutralize the virus by interfering with E2-CD81 interaction. In addition to nAbs recognizing E1 or E2 alone, human mAbs targeting an epitope shared by both proteins on the E1E2 heterodimer have also been identified [32]. The E2C45 region identified in the present work is distinct from the previously reported neutralizing epitopes.

Improvement in the antigenicity of E2C45 peptide capable of eliciting a stronger nAb response could also help in its application potential as a prophylactic agent. This peptide exhibits the ability to generate cross-reactive nAbs since we have shown that anti-E2C45 antibody inhibits the entry of JFH1 virus, which is genotype 2a.

The mAbs generated against the peptide E2C45 were more effective against JFH1 gt2 virus (up to 95 %) compared to patient serum-derived gt3 virus in the entry inhibition assay. Since the E2C45 was derived from gt3 virus, this result was unexpected. This could be due to the fact that the HCVcc (JFH1) is quite different from patient serum-derived natural virus in terms of efficiency/mechanism of internalization. Further experiments are necessary to address this issue.

The extent of inhibition by the mAbs was found to be more pronounced in Huh7 cells compared to CHO cells expressing CD81. This could be due to the fact that levels of expression of CD81 could be low compared to the abundance of this receptor in Huh7 cells.

In summary, we have identified a novel epitope at the C terminus of E2 that induces potent nAbs in HCV-infected patients who recover from infection. This is the first report on the discovery of a synthetic peptide (E2C45) that corresponds to the novel epitope which induced potent nAbs in mice following immunization with peptide-conjugate vaccine. The human antibodies (IgG) and the mouse antibodies neutralized HCV in a cell culture system in vitro and demonstrated cross genotype neutralizing activity.

**METHODS**

**Ethical clearance**

All animal experiments have been approved by the ‘Institutional Animal Ethics Committee’. Female BALB/c mice were selected from an inbred colony and maintained under standard conditions. Mice were fed with pelleted food and water ad libitum. Serum samples were collected from patients at Yashoda hospital, Hyderabad, India, after clearance by the human ethics committee.

**Cell culture**

Huh7 and Huh7.5 cells were maintained in Dulbecco’s modified Eagle medium (DMEM; Sigma) supplemented with 10 % FBS at 37 °C under 5 % CO2. Huh7.5 cells were a generous gift from Dr C. M. Rice (Apath LLC, St Louis, MO, USA). Sf21 cells were maintained in TC100 insect cell medium (Sigma) with 10 % FBS at 26 °C. Hybridoma cells were maintained in IMDM media (Sigma) supplemented with 10 % FBS, sodium bicarbonate, β-mercaptoethanol, glutamate, penicillin (100 U ml−1) and streptomycin (100 µg ml−1).

**Generation and purification of baculovirus-based HCV-LPs**

The nucleotide sequence encoding HCV core-E1-E2 (gt3a) was amplified from RNA isolated from the blood of an HCV-infected patient and cloned in pGEMT-Easy vector (accession nos.: core, GU172376; E1E2, GU172375) and HCV-LP generated as described earlier [45, 46]. Similarly, the core-E1-E2 of gt1b was amplified from replicate Con1FL (accession no. AJ238799) [47] and HCV-LP for gt1b was generated as mentioned earlier [45, 46]. To generate HCV-LPs, Sf21 cells were infected with recombinant baculovirus (at an m.o.i. of 5). After 72 h, cell pellets were washed three times with PBS (50 mM phosphate buffer, pH 7.2, containing 150 mM NaCl) and were resuspended in lysis buffer (50 mM NaCl, 50 mM Tris, 0.5 mM EDTA, 1 mM PMSF, 0.1 % Nonidet P-40 and 0.25 % protease inhibitors) using a homogenizer. The lysate was centrifuged and HCV-LPs were purified using sucrose gradient ultracentrifugation [45, 46]. The HCV-LPs (both gt3a and gt1b) were characterized by ELISA and confirmed by electron microscopy [45, 46].

**IgG purification from human serum**

To demonstrate the inhibition of HCV-LP binding to Huh7 cells, IgG was purified from HCV-positive and HCV-negative control sera using a Protein G HP Spin Trap/Ab Spin Trap (GE Healthcare). Briefly, a Spin Trap Protein G column was generated as mentioned earlier [45, 46]. To generate HCV-LPs, Sf21 cells were infected with recombinant baculovirus (at an m.o.i. of 5). After 72 h, cell pellets were washed three times with PBS (50 mM phosphate buffer, pH 9.0). Serum (200 µl) was applied to the column, incubated for 5 min, eluted using elution buffer (pH 2.5) buffer and then neutralized using 10 µl of neutralizing buffer, as per the manufacturer’s protocol. Purified IgG was analysed for the presence of anti-HCV antibodies using an HCV-LP (gt3a and gt1b)-based ELISA. The concentration of IgG was determined by a Bradford assay.

**Flow cytometry analysis to study binding of HCV-LP to Huh7 cells**

HCV-LPs (gt3a and 1b) were labelled with Alexa Fluor 488 (Invitrogen) pre-incubated with antibodies and the complex was added to Huh7 cells. Binding of the VLPs was
monitoring by flow cytometry as described earlier [45, 46]. Briefly, HCV-LPs of both genotypes were labelled with Alexa Fluor 488 using a gel exclusion chromatography column (Sigma Aldrich). The labelled VLPs were incubated with 1:100 dilutions of serum for 1 h at 37 °C. Huh7 cells (5×10^5) were added to the mixture of HCV-LPs and antibody in the presence of DMEM and 25 mM of HEPES buffer (final volume, 100 µl) and incubated for 3 h at room temperature. Unbound HCV-LP–antibody complexes were removed by washes with 25 mM HEPES in DMEM. Cell-bound fluorescence was analysed using an FACS calibur flow cytometer (Becton Dickinson) using Cylogic software to calculate the median fluorescence intensity (MFI) of the cell population, which directly relates to the surface density of Alexa-labelled HCV-LPs bound to hepatocytes. The MFI values of cells with or without HCV-LPs were compared and per cent binding was determined from the equation:

\[
\% \text{binding of VLP to cells} = \frac{\text{experimental MFI} - \text{negative control (only cells) MFI}}{\text{positive control MFI} - \text{negative control (only cells) MFI}} \times 100.
\]

**Peptides**

Peptides were obtained through BEI Resources, NIAID, NIH, USA: peptide array, HCV, K3a/650, core protein (NR-4061), E1 protein (NR-4062) and E2 protein, NR-4063. Each peptide comprised 16 aa with 11 aa overlap between consecutive peptides. The 45-mer peptide (E2C45) corresponding to position 634–679 of E2 protein was obtained from GenMed.

**ELISA**

Microtitre ELISA plates (Nunc) were coated overnight with antigen (HCV-LP) (5 µg ml^-1 in PBS) or peptides (10 µg ml^-1 in PBS) followed by blocking of unoccupied sites with 0.5% gelatin in PBS. The plates were incubated with different serum samples (HCV-infected patients and healthy control) and culture supernatant samples of hybridoma generated to E2C45. After three washes with PBS containing 0.05% Tween 20, the plates were incubated with rabbit anti-mouse IgG-HRP conjugate (DAKO) for 1 h. The bound-peroxidase activity was detected using tetramethyl benzidine in citrate buffer, pH 5.0, and 0.03% H2O2. The reaction was stopped with 1 M H2SO4, and absorption at 450 nm was measured in an ELISA plate reader (Spectramax; Molecular Devices).

**Immunization of mice and establishment of hybridomas**

E2C45 peptide was conjugated to BSA, emulsified with Freund’s adjuvant and administered to 6- to 8-week-old female BALB/c mice (30 µg per mouse, subcutaneously). After three doses of the immunogen at three weekly intervals, the mice were rested for a month. The mice were finally injected intraperitoneally with 100 µg of the antigen in saline and 4 days later the animals were killed. The spleens were excised, and the splenocytes were fused with Sp2/0 mouse myeloma cells using polyethylene glycol 4000 (Merck). Hybridomas were established using protocols standardized in our laboratory. mAbs were purified from the culture supernatant on protein A-Sepharose beads [45].

**Generation of CHO cells stably expressing CD81 receptor and antibody-mediated inhibition of HCV-LP 3a binding to host cells**

As described earlier [38], the human CD81 gene construct was subcloned in the pcDNA3.1(−) vector between BamHI and HindIII restriction sites. CHO cells were transfected with either pcDNA (CHO-pcDNA) or pcDNA-CD81 (CHO-CD81) using a Bio-Rad gene pulsar Xcell (250 V and 950 µF followed by selection of transfected cells on Geneticin; Calbiochem). For inhibition of binding of HCV-LPs to CHO-CD81 cells, purified mAbs (A8A11, C10E8, E1B11 and E3D8) at 100 µg ml^-1 were incubated with Alexa-488 labelled HCV-LPs for 1 h, then added to CHO-CD81 cells (5×10^5) and incubated for 1 h. The binding of labelled VLPs was observed by flow cytometry.

**In vitro transcription of viral RNA**

The pJFH1 construct (a generous gift from Dr Takaji Wakita, National Institute of Infectious Diseases, Tokyo, Japan) was linearized with XbaI. HCV RNA was synthesized from linearized pJFH1 template using Ribomax Large Scale RNA Production System-T7 according to the manufacturer’s instructions (Promega) [48].

**Transfection and generation of HCV-JFH1 virus**

Huh7.5 cells were transfected with in vitro synthesized HCV-JFH1 RNA transcript using Lipofectamine 2000 (Invitrogen) in Opti-MEM medium (Invitrogen). Infectious JFH1 virus particles were generated as described previously [49]. Uninfected Huh7.5 cells were used as mock control.

**Virus neutralization assay**

Polyclonal antibodies from HCV-infected patient serum and mice injected with E2C45 as well as mAbs generated against E2C45 peptide were tested for their ability to neutralize virus infectivity. Huh7.5 cells were seeded in 24-well plates 16 h prior to infection. JFH1 virus was incubated with serial dilutions of antibodies at 37 °C for 1 h. The antibody–virus mixture was then transferred on the cells. Infectivity was analysed 3 days post-infection by qRT-PCR for HCV-negative strand detection [49].

**Quantification of viral RNA**

Viral RNA was quantified by real-time RT-PCR analysis. Cells were harvested 3 days (for HCV-negative sense strand detection) post-infection and total RNA was isolated, which was reverse transcribed with HCV 149 forward (for HCV-negative sense) and GAPDH reverse primer using RevertAid reverse transcriptase (Thermo Scientific). The resulting cdNA was amplified for HCV IRES and GAPDH (internal control) using the ABI real-time PCR System (ViiA7; Applied Biosystems).

Sequences of primers used are listed below:

1. HCV149F: 5′TGCCGAACCGTGAGTACA3′
Generation of patient serum-derived HCV gt3 virus

For preparing serum-derived gt3 virus, we followed the method as reported earlier [45, 46]. Briefly, 200 µl serum from an HCV gt3-infected patient (viral titre of 1 × 10^8 IU ml⁻¹) was used to infect 0.8 × 10^6 Huh7.5 cells in a 60 mm dish in serum-free medium. Twenty-four hours post-infection, cells were changed to complete medium. The dish was washed twice with PBS and stored at −80 °C. The viral supernatant was collected, filtered through a 0.2 µm filter (Millipore) and concentrated using the Maxisorp 100 kDa Centricon (Pall Lifesciences). The concentrated viral supernatant was supplemented with 20% FBS and stored at −80 °C. The infected supernatant was passaged further in Huh7.5 cells and concentrated to increase the titre of the virus. The viral replication was determined by qRT-PCR as well as by immunofluorescence assays (Fig. S1). The negative strand RNA level was quantified from the infected cells by qRT-PCR at different time points (24, 48, 72 and 96 h). HCV E2 protein was detected by an immunofluorescence assay using the combination of the mAbs (A8A11 and C10E8) using a similar protocol as described in the previous section. The concentrated supernatant was then used as a source of gt3 virus for the neutralization experiment using the same procedure as followed above for JFH1 virus.

Virus neutralization assay with HCV gt3 virus

For preparing serum-derived gt3 virus, we followed the method as reported earlier [46]. The gt3 virus was used for the neutralization experiment with purified mAbs (A8A11 and C10E8 or in combination) using the same procedure as followed for JFH1 virus. Briefly, viral particles were incubated with two different concentrations of the mAbs (50 and 100 µg ml⁻¹) each) at 37 °C for 1 h, following which the antibody–virus mixture was added to the cells. Seventy-two hours post-infection, total RNA was isolated and reverse transcribed with HCV IRES 5’ forward primer (and GAPDH 3’ reverse primer) to detect HCV negative strand RNA level. HCV IRES and GAPDH (internal control) was amplified from the resulting cDNA by qRT-PCR using SYBR green PCR master mix (Thermo Scientific) in an ABI ViiA7 real-time PCR system (Applied Biosystems).

Infectious foci reduction assay

JFH1 virus was incubated with different concentrations of the individual mAbs or their combinations (50 and 100 µg ml⁻¹) in serum-free medium at 37 °C for 1 h. The samples were then added to 0.08 × 10^6 Huh7.5 cells seeded in a 24-well plate 16 h before infection and incubated for 4 h. Then, 72 h post-infection, cells were washed with PBS and fixed with 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton-X-100, washed with PBS and blocked with 3% BSA. Next, cells were probed with 50 µg ml⁻¹ of the combinations of the mAbs A8A11 and C10E8, washed with PBS and further probed with Alexa Fluor 488-conjugated anti-mouse secondary antibody (Invitrogen). Cells were further washed with PBS, stained with DAPI and mounted on the cover slips with Fluoroshield mounting medium (Sigma). Images were taken in a confocal microscope and the images were analysed using Zen software.


43. Vieyres G, Dubuisson J, Patel A. Characterization of antibody-mediated neutralization directed against the hypervariable region


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