Supplementary Materials and Methods

Analysis of HCVpp Envelope Glycoprotein Expression

Expression of HCV glycoproteins was characterized in HEK 293T producer cells and HCVpp purified through a 20% sucrose cushion ultracentrifugation as described.[1] Immunoblots of HCV glycoproteins were performed using anti-E1 11B7 and anti-E2 AP33 mAbs as described.[2]

Cellular Binding of Envelope Glycoproteins

Envelope glycoprotein-expressing HEK 293T cells were lysed in phosphate-buffered saline by 4 freezing and thawing cycles. Cell debris and nuclei were removed by low-speed centrifugation and supernatants containing native intracellular E1E2 complexes were used for binding studies. Huh7.5.1, shCD81-, or shCD13-Huh7.5 cells (2×10^5 cells per well) were seeded in 96-well plates. After incubation with lysates containing patient-derived E1E2 proteins, Huh7.5.1 target cells were first incubated with mAb AP33 (10 μg/mL) and then with phycoerythrin-conjugated anti-mouse Ab (5 μg/mL, BD Biosciences). Bound E2 was analyzed by flow cytometry as described.[3]

Construction of Plasmids for Production of Chimeric HCVcc Expressing Patient-Derived Envelopes

Genotype 1 JFH-based HCVcc chimeras expressing the structural proteins of patient-derived viruses were produced as previously described for Con1/C3-JFH1-V2440L.[4,5] Briefly, the complementary DNA region encoding for the HCV core to the first transmembrane domain of NS2 (C3 junction site) from variant VL was inserted into pFK-Con1/C3-JFH1-V2440L using fusion polymerase chain reaction with Pfu DNA polymerase (Agilent Technologies, Massy, France) and standard cloning procedures using appropriate restriction sites including BsmI and AvrII. The obtained construct was designated *VL/JFH1*. The VL/JFH1 encoding sequence was used as a template to insert individual and combined mutations using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) as described previously.[1]

Galanthus nivalis *Capture Enzyme-Linked Immunosorbent Assay*

Binding of HMAb CBH-23 to viral envelopes was analyzed using an enzyme-linked immunosorbent assay with HCVpp as a capture antigen as described.[6] HCVpp expressing the E1E2 glycoproteins of HCV variants or control pseudoparticles with absent HCV envelope glycoprotein expression were partially purified and enriched through ultracentrifugation as described.[1] Purified particles were quantified as described previously.[1] Partially purified HCVpp or control pseudoparticles were captured onto *Galanthus nivalis* (GNA)-coated microtiter plates as described.[6] Soluble E2 (derived from strain HCV-H77 and expressed in 293T cells as described previously[3]) was used as a positive control for antibody binding. Neutralizing human anti-E2 antibody CBH-23 (25 μg/mL diluted in phosphate-buffered saline) then was added to captured HCVpp or soluble E2 (1 h at room temperature). After washing and removal of nonbound antibody, mAb binding to HCV envelopes was detected using horseradish-peroxidase anti-human IgG (GE Healthcare, Orsay, France) at a concentration of 1/3000 for 1 hour at room temperature, followed by incubation with 1-step Turbo TMB–enzyme-linked immunosorbent assay (Thermo Fisher Scientific, Illkirch, France) for color development. Absorbance was measured at 450 nm using a microplate reader Softmax program (Molecular Devices, Sunnyvale, CA).

Bioinformatics

Multiple sequence alignment of complete E2 proteins was performed using the European HCV databases (http://euhcvdb.ibcp.fr).[7] Two amino acid repertoires were computed with all E2 sequences of provisional/confirmed genotype 1b using the *ComputeRepertoire* tool as part of the euHCVdb *Extract* tool (http://euhcvdb.ibcp.fr).

Results

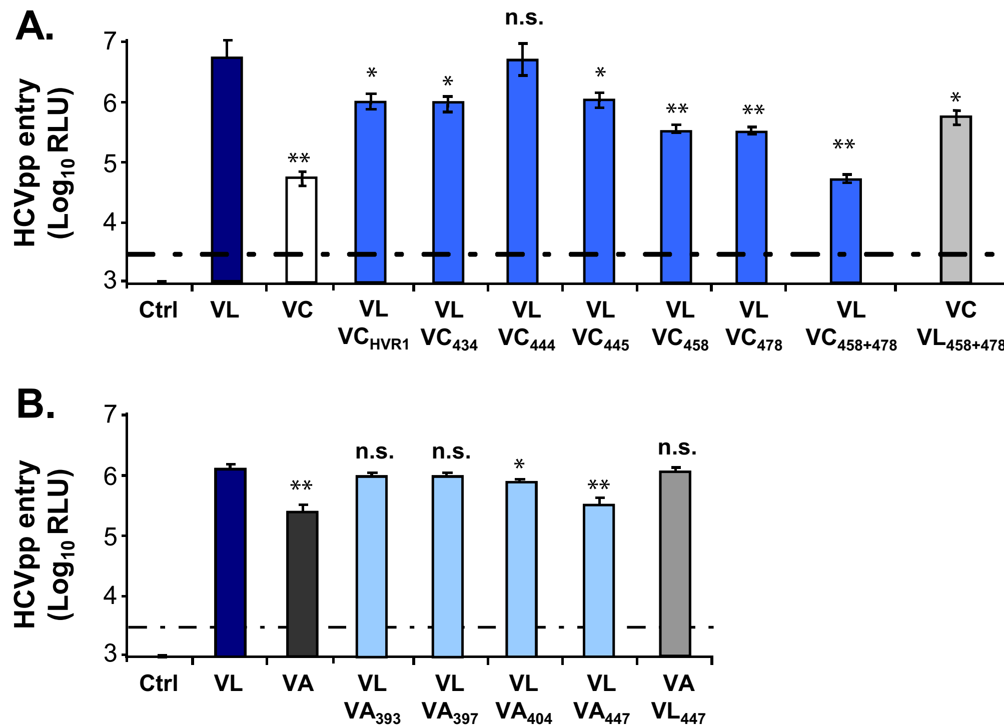
Prevalence of the Identified Mutations in a Large Genomic Database of Viral Isolates

Bioinformatic sequence analysis of a large panel of 2074 HCV strains within the European HCV database further supports the potential relevance of the identified positions for pathogenesis of HCV infection in general.[7] Residues F, S, and R are observed much more frequently at positions 447, 458, and 478 than L, G, and C. F and S are the most predominant residues at positions 447 and 458 in the large majority of 1b strains, respectively (F447 all, 98.4%; 1b, 96.2%; S458 all, 94%; 1b, 90.3%; Supplementary Figure 5). The position 478 is variable but R (all, 2.4%; 1b, 10.8%) is more frequent than C (all, 0.2%; 1b, 0.9%) (Supplementary Figure 5). The high prevalence of identified residues supports their functional relevance for virus survival and selection because more structurally and functionally relevant residues will be observed more frequently. These data suggest that the epitope containing the identified residues at positions 447, 458, and 478 is responsible not only for viral evasion from autologous antiviral antibodies during LT but also may contribute to viral evasion in chronic HCV infection in general.

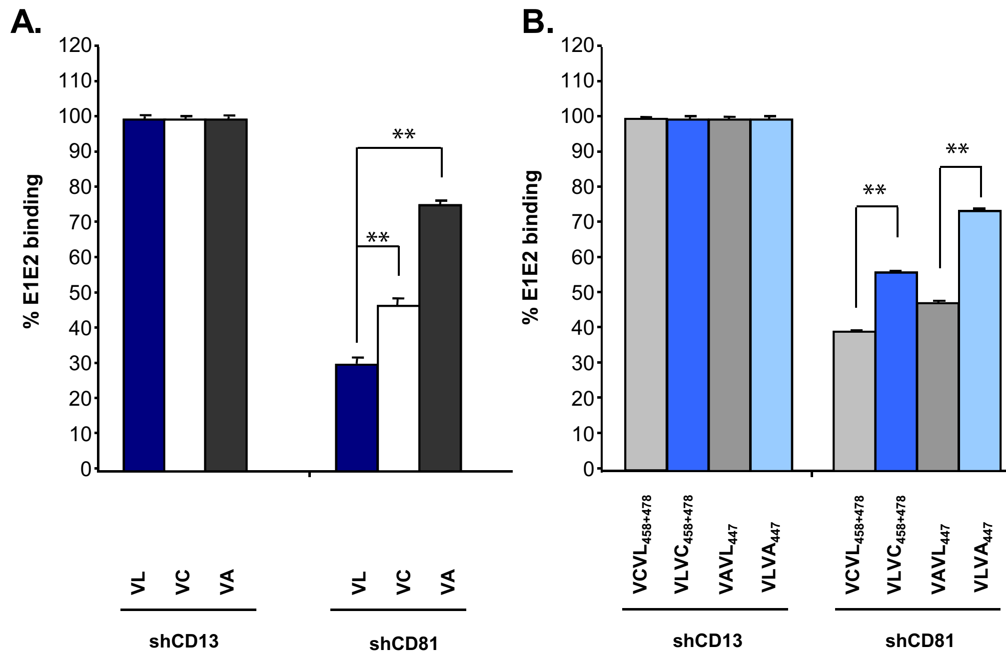
References

1. Fafi-Kremer S, Fofana I, Soulier E, et al. Enhanced viral entry and escape from antibody-mediated neutralization are key determinants for hepatitis C virus re-infection in liver transplantation. J Exp Med 2010;207:2019–2031.
2. Pestka JM, Zeisel MB, Blaser E, et al. Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. Proc Natl Acad Sci U S A 2007;104: 6025–6030.

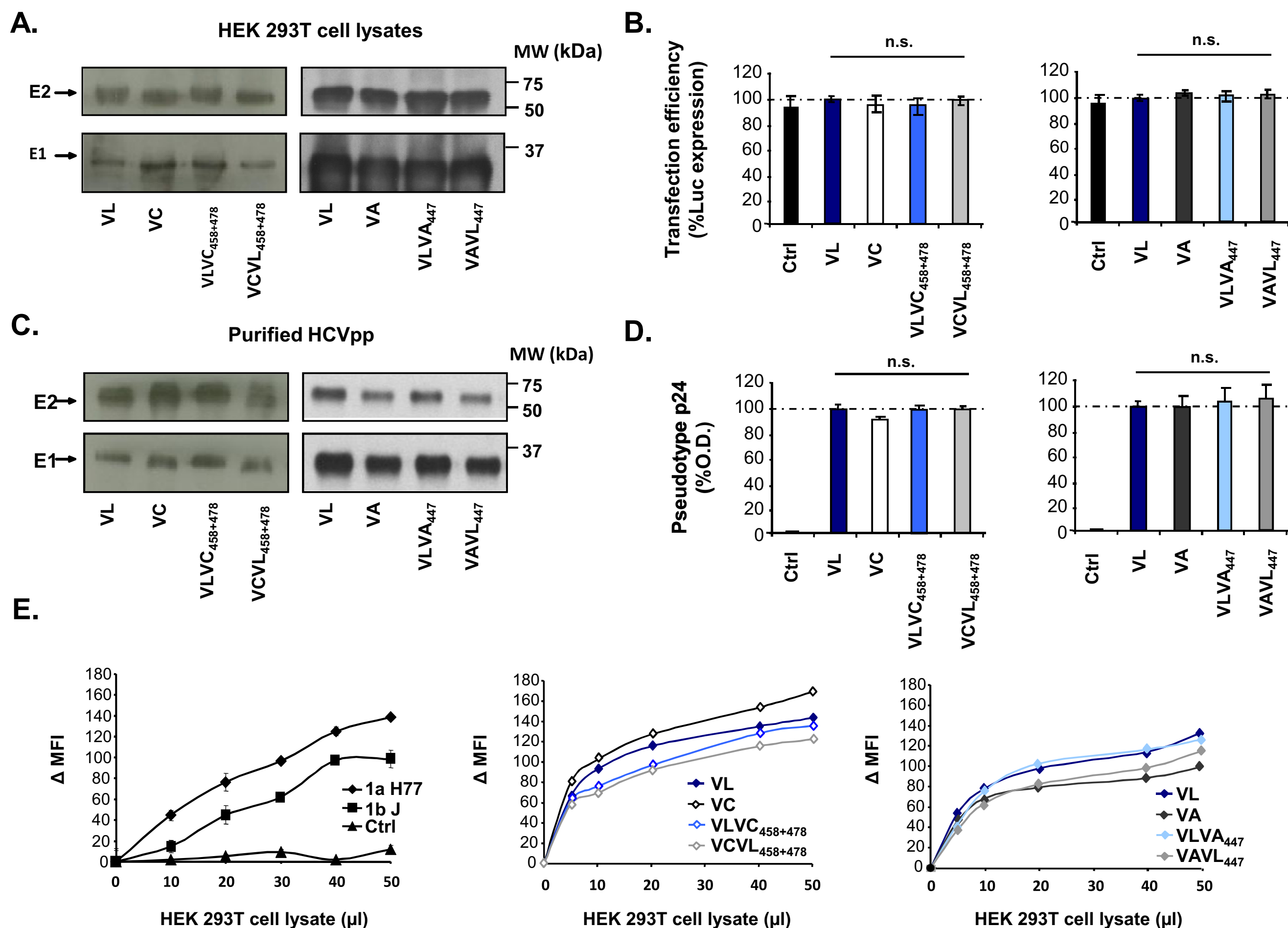
3. Krieger SE, Zeisel MB, Davis C, et al. Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by neutralization of E2-CD81-claudin-1 associations. Hepatology 2010;51:1144–1157.
4. Pietschmann T, Kaul A, Koutsoudakis G, et al. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc Natl Acad Sci U S A 2006;103:7408–7413.
5. Kaul A, Woerz I, Meuleman P, et al. Cell culture adaptation of hepatitis C virus and in vivo viability of an adapted variant. J Virol 2007;81:13168–13179.
6. Wang Y, Keck ZY, Saha A, et al. Affinity maturation to improve human monoclonal antibody neutralization potency and breadth against hepatitis C virus. J Biol Chem 2011;286:44218–44233.
7. Combet C, Garnier N, Charavay C, et al. euHCVdb: the European hepatitis C virus database. Nucleic Acids Res 2007;35:D363–D366.
8. Owsianka A, Tarr AW, Juttla VS, et al. Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. J Virol 2005;79:11095–11104.
9. Haberstroh A, Schnober EK, Zeisel, et al. Neutralizing host responses in hepatitis C virus infection target viral entry at post-binding steps and membrane fusion. Gastroenterology 2008;135:1719–1728.
10. Hadlock KG, Lanford RE, Perkins S, et al. Human monoclonal antibodies that inhibit binding of hepatitis C virus E2 protein to CD81 and recognize conserved conformational epitopes. J Virol 2000;74:10407–10416.
11. Keck ZY, Li SH, Xia J, et al. Mutations in hepatitis C virus E2 located outside the CD81 binding sites lead to escape from broadly neutralizing antibodies but compromise virus infectivity. J Virol 2009;83:6149–6160.
12. Dimitrova M, Affolter C, Meyer F, et al. Sustained delivery of siRNAs targeting viral infection by cell-degradable multilayered polyelectrolyte films. Proc Natl Acad Sci U S A 2008;105:16320–16325.
13. Lupberger J, Zeisel MB, Xiao F, et al. EGFR and EphA2 are hepatitis C virus host entry factors and targets for antiviral therapy. Nat Med 2011;17:589–595.
14. Bartosch B, Vitelli A, Granier C, et al. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J Biol Chem 2003;278:41624–41630.



Supplementary Figure 1. Actual viral infectivity of HCVpp derived from variants VL, VC, and VA shown as relative light units (RLU) of luciferase reporter gene expression. (*A* and *B*) Comparative analysis of viral entry of HCVpp shown in Figure 1. Results are expressed in RLUs plotted in a logarithmic scale. The threshold for a detectable infection in this system is indicated by *dashed lines*. The detection limit for positive luciferase reporter protein expression was 3×10^3 RLU/assay, corresponding to the mean ± 3 standard deviations of background levels (ie, luciferase activity of naive noninfected cells or cells infected with pseudotypes without HCV envelopes).[1,12,13] Background levels of the assay were determined in each experiment. Means ± standard deviation from at least 4 independent experiments performed in triplicate are shown. Significant differences in HCVpp entry VC, VA, and VL wild-type and mutant variants are indicated ($*P \leq .05$; $**P < .001$). Ctrl, control; HVR, hypervariable region; V, viral variant.



Supplementary Figure 2. Positions 447, 458, and 478 modulate binding of envelope glycoproteins to CD81 expressed at the cell surface. Binding of native E1E2 complexes expressed from patient-derived complementary DNAs to Huh7.5 cells with silenced CD81 expression (described in Figure 3) was detected by flow cytometry. Results are expressed as the percentage of E1E2 binding compared with shCD13-Huh7.5 control cells. Means ± standard deviation from 3 independent experiments performed in triplicate are shown. Significant differences in binding between variants are indicated (**$P < .001$).

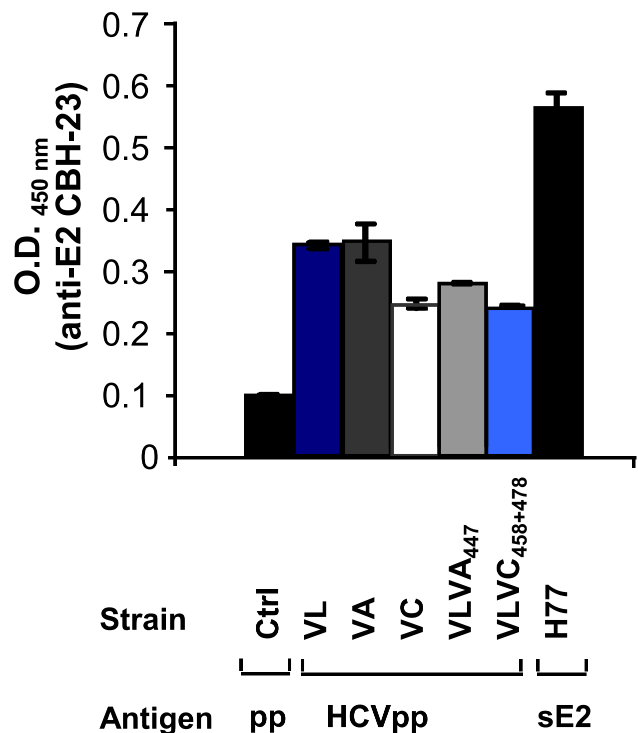


Supplementary Figure 3. Differences in viral entry are not caused by impaired HCVpp production. (*A*) Analysis of envelope glycoprotein expression. Protein expression was analyzed by immunoblotting as described in the Materials and Methods section. Molecular markers (in kilodaltons) are indicated on the *right*. (*B*) Transfection efficiency during HCVpp production. Transfection efficiency was analyzed for each variant and quantified by determining luciferase expression in HEK 293T producer cells expressed as a normalized percentage compared with control transfected cells. (*C*) Envelope glycoprotein expression in HCVpp. HCVpp were purified as described previously[1,2] and subjected to immunoblot as described in panel *A*. (*D*) Lentiviral p24 antigen expression was analyzed by enzyme-linked immunosorbent assay (ELISA) and is indicated as optical density (OD) values at 450 nm. (*E*) Cellular binding of E2 derived from patient-derived or H77 and HCV-J strains. Binding of native E1E2 complexes to Huh7.5.1 cells was detected as described in Supplementary Materials and Methods. Results are expressed as delta mean fluorescence intensity (ΔMFI) ± standard deviation. One representative experiment of 3 is shown. Da, dalton; MW, molecular weight.

Supplementary Table 1. Neutralization of Patient-Derived and Chimeric HCVpp by Monoclonal Anti-Envelope Antibodies

Antibody	Reference	Epitope, amino acid	HCVpp entry, % VL	VC	$VCVL_{458+478}$	$VLVC_{458+478}$	VA	$VAVL_{447}$	$VLVA_{447}$
AP33	8	412–423	6 ± 3	12 ± 1	3 ± 1	11 ± 5	2 ± 1	5 ± 1	3 ± 1
IGH461	9	436–448	58 ± 4	56 ± 8	51 ± 7	53 ± 3	55 ± 2	56 ± 6	52 ± 7
16A6	9	523–530	76 ± 10	74 ± 8	83 ± 9	82 ± 2	73 ± 9	74 ± 4	81 ± 9
CBH-2	10	Domain B, conformational 431, 523–540	60 ± 5	8 ± 5	65 ± 6	9 ± 5	39 ± 8	61 ± 4	39 ± 10
CBH-5	10	Domain B, conformational 523–540	71 ± 2	10 ± 4	73 ± 7	8 ± 1	36 ± 5	59 ± 7	47 ± 8
CBH-23	Keck and Foung, unpublished data	Domain C, conformational	97 ± 9	21 ± 6	98 ± 13	14 ± 3	32 ± 7	53 ± 12	44 ± 3
HC-1	11	Domain B, conformational 523–540	73 ± 5	31 ± 9	81 ± 10	27 ± 9	2 ± 1	2 ± 1	77 ± 1

NOTE. HCVpp produced from isolates shown in Figure 1 were incubated with mAbs (10 μg/mL) for 1 hour at 37°C. HCVpp-antibody complexes then were added to Huh7.5.1 cells. Viral epitopes targeted by the respective antibody, percentage of HCV entry in the presence of antibody (strains VL, VC, $VCVL_{458+478}$, $VLVC_{458+478}$, VA, $VAVL_{447}$, and $VLVA_{447}$), and source or reference of antibody are shown. Means ± standard deviation from at least 3 experiments, each performed in triplicate, are shown.
V, viral variant.



Supplementary Figure 4. Binding of neutralizing anti-E2 HMAb CBH-23 to patient-derived envelope glycoproteins expressed on HCVpp as capture antigens in an enzyme-linked immunosorbent assay (ELISA). HCVpp expressing envelope glycoproteins of variants VL, VA, VC, $VLVA_{447}$, and $VLVC_{458+478}$ were used as capture antigens on GNA-coated ELISA plates. Control (Ctrl) pseudoparticles with absent HCV envelope glycoprotein expression and recombinant soluble E2 (sE2 derived from strain H77)[14] served as negative and positive controls, respectively. Anti-E2 CBH-23 reactivity was detected as described in the Supplementary Materials and Methods section and is indicated as optical density (OD) values at 450 nm. Means ± standard deviation from 1 representative experiment are shown.

Supplementary Table 2. Characteristics of Patients and Viruses Used for Neutralization Studies

					HCVpp neutralization titer, 1/dilution		
Patient number	Age, y	Sex	Genotype	Viral load, IU/mL	VL	VC	VA
1	65	M	1b	2.29×10^5	100	100	100
2	27	F	1b	9.7×10^4	100	3200	200
3	31	F	1b	1.53×10^5	400	3200	400
4	47	M	3a	1.02×10^6	20	20	100
5	58	M	1b	1.15×10^6	100	3200	200
6	72	M	1b	1.50×10^6	20	200	100
7	51	M	4	4.38×10^6	20	20	20
8	69	F	1b	9.7×10^5	20	400	100
9	36	F	1	1.29×10^5	800	1600	100
10	46	M	1a	1.05×10^6	100	800	100
11	55	M	1a	1.54×10^6	400	3200	200
12	56	M	4c/4d	2.41×10^4	20	800	200
13	56	F	4a	1.09×10^6	100	400	400
14	59	F	1b	3.54×10^5	200	800	200
15	62	M	1a	3.37×10^6	20	20	20
16	50	M	4a	1.48×10^6	20	200	20
17	46	M	4a	4×10^5	20	200	100
18	70	F	1b	1.3×10^6	100	800	20
19	77	F	1b	6.2×10^4	20	100	100
20	61	F	1b	2.58×10^4	200	800	200
21	46	F	1b	2.11×10^5	100	400	800
22	36	M	1a	2.04×10^6	20	200	400
23	52	F	4a	9.12×10^5	20	3200	400
24	54	M	1a	9.77×10^5	100	800	200
25	54	M	1b	1.12×10^6	20	100	200
26	54	F	1a	3.38×10^6	20	400	20
27	47	M	3a	6.16×10^5	100	3200	3200
28	43	M	1a	5.75×10^6	20	800	200
29	51	M	4a	1.44×10^6	100	400	400
30	54	M	2c	4.67×10^5	100	100	3200
31	51	M	1a	6.16×10^6	100	400	100
32	39	M	4a	1.12×10^6	20	200	800
33	62	F	4f	2.88×10^6	20	800	20
34	46	M	4k	3.54×10^5	20	20	100
35	42	M	1a	9.54×10^5	400	800	400
36	54	M	2c	4.67×10^5	200	3200	100
37	34	M	3a	3.23×10^6	20	20	100
38	47	M	3a	7.94×10^4	20	400	20
39	30	F	1b	1.00×10^6	20	200	400
40	47	F	1b	2.29×10^6	100	400	200
41	52	M	1a	1.73×10^6	200	3200	400
42	34	M	1b	1.45×10^6	3200	3200	200
43	46	M	1a	4.34×10^6	200	800	400
44	66	F	1b	3.89×10^5	200	1600	200
45	29	F	1a	1.08×10^5	400	400	200
46	45	M	3a	2.78×10^5	20	200	200
47	65	F	4f	1.46×10^6	20	3200	20
48	55	M	1a	8.81×10^6	20	800	100
49	53	M	1a	1.15×10^6	100	100	100
50	40	M	3a	2.46×10^6	100	3200	200
51	48	F	1a	1.00×10^5	20	800	20
52	37	M	1a	5.08×10^6	20	400	200
53	47	M	3a	6.8×10^6	100	1600	400
54	37	M	1a	1.84×10^6	800	800	200
55	65	F	1b	2.18×10^6	100	100	800
56	45	F	1a	3.93×10^6	1600	1600	400
57	49	M	4a	2.06×10^6	800	3200	200
58	30	M	1b	7.21×10^5	100	800	200
59	31	M	3a	6.66×10^6	100	200	200
60	37	M	1a	6.70×10^6	20	100	100

Supplementary Table 2. Characteristics of Patients and Viruses Used for Neutralization Studies

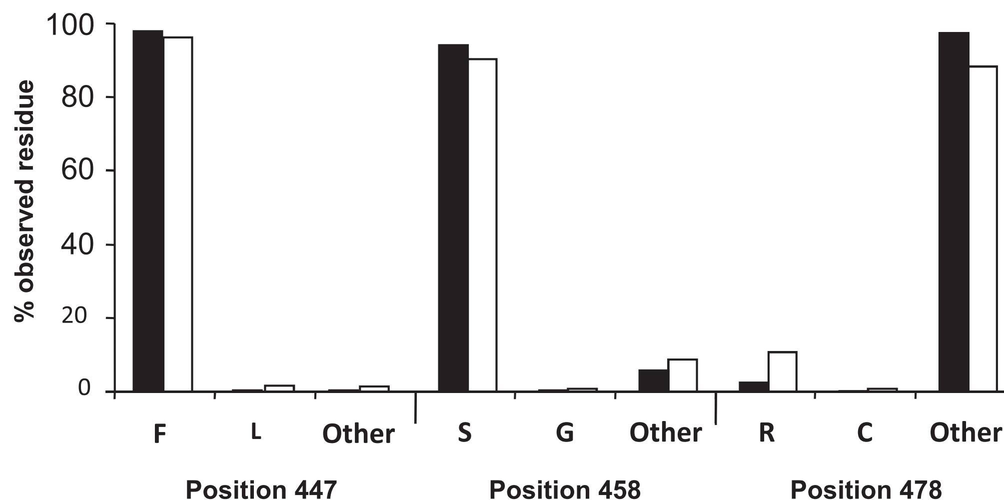
					HCVpp neutralization titer, 1/dilution		
Patient number	Age, y	Sex	Genotype	Viral load, IU/mL	VL	VC	VA
61	49	M	1a	3.16×10^5	20	800	20
62	43	M	1	6.83×10^5	20	20	20
63	69	M	1b	4.7×10^5	20	20	200
64	48	M	1a	3.28×10^6	20	3200	100
65	46	M	3a	8.55×10^5	20	800	100
66	51	M	1b	1.07×10^6	20	200	1600
67	43	M	1b	4.27×10^5	20	100	800
68	36	M	3a	1.14×10^6	20	800	20
69	53	F	1b	3.06×10^5	20	400	20
70	24	F	3a	1.29×10^6	20	20	20
71	63	M	1b	3.01×10^6	100	200	100
72	44	M	1	1.10×10^5	20	3200	200
73	28	M	3a	1.85×10^6	20	3200	20
74	54	M	1b	1.29×10^5	20	3200	20
75	17	F	1b	2.41×10^5	20	20	200
76	40	M	3a	1.26×10^6	20	20	100
77	35	M	1b	8.89×10^5	20	20	800
78	36	F	6a	1.4×10^7	20	100	400
79	70	F	1b	1.13×10^5	100	100	400
80	62	M	1a	2.68×10^6	100	200	20
81	70	M	1b	2.85×10^5	20	200	3200
82	63	M	1b	1.95×10^5	200	400	400
83	33	M	1a	1.76×10^6	100	200	800
84	35	M	1a	2.78×10^6	20	20	200
85	60	F	1	6.39×10^5	20	200	100
86	57	M	3a	1.22×10^6	200	3200	400
87	60	M	1	3.6×10^6	100	3200	20
88	49	M	4	2.24×10^6	20	1600	20
89	37	M	4	9.35×10^5	100	800	100
90	55	M	1a	3.77×10^6	20	3200	100
91	47	M	1a	2.36×10^6	20	1600	20
92	72	M	3a	3.83×10^5	20	400	20
93	79	M	1b	2.81×10^5	100	1600	100
94	58	F	1b	6.58×10^5	100	3200	200
95	50	M	3a	6.07×10^5	20	3200	100
96	67	F	1b	4.13×10^5	100	800	20
97	49	M	3a	5.22×10^5	200	400	200
98	53	F	1b	2.31×10^6	20	400	1600
99	37	M	1a	1.87×10^5	100	3200	200
100	54	F	4a	9.23×10^5	20	200	100
101	39	M	1a	1.76×10^5	100	800	200
102	51	F	2b	1.10×10^6	100	3200	800

NOTE. HCVpp were incubated with anti-HCV–positive sera from 102 patients with chronic HCV infection (ClinicalTrial.gov identifier NCT00638144). Patient number, age, sex, viral genotype, and load in serum are indicated. HCVpp-antibody complexes were added to Huh7.5.1 cells and infection was analyzed as described in Figure 4. Calculation of neutralization and determination of background and thresholds for neutralization were performed as described in Figure 6. Neutralization titers obtained by end point dilution are indicated for each variant. Means from at least 3 independent experiments, each performed in triplicate, are shown.
V, viral variant.

Supplementary Table 3. HCVcc Neutralization Titers

Patient number	HCVcc neutralization titer, 1/dilution		
	VL	$VLVC_{458+478}$	$VLVA_{447}$
11	400	1600	800
28	20	1600	800
33	20	400	400
35	400	1600	1600
36	200	1600	3200
45	800	1600	800
65	20	1600	1600
66	20	3200	800
68	20	1600	1600
94	100	3200	800
98	100	800	3200
99	100	3200	1600

NOTE. Results were confirmed using chimeric HCVcc expressing the HCV envelope glycoproteins depicted in Figure 7 and using 12 representative sera from patients. Neutralization assays were performed using a similar protocol as described in Supplementary Tables 2 and 3. Means from at least 3 independent experiments, each performed in triplicate, are shown.
V, viral variant.



Supplementary Figure 5. Distribution of residues at positions 447, 458, and 478 of HCV E2 sequences in the European HCV databases. Distribution of residues at positions 447, 458, and 478 for HCV complete E2 sequences from all subtypes (black) and from subtype 1b only (white) within the European Hepatitis C Virus databases[7] (available: http://euhcvdb.ibcp.fr). F and S are the predominant residue at positions 447 and 458 (F447, 98.4%; 1b, 96.2%; S458 all, 94%; 1b, 90.3%). The position 478 is variable (it belongs to HVR2) but R (all, 2.4%; 1b, 10.8%) is more frequent than C (all, 0.2%; 1b, 0.9%).