Hepatitis C Virus–Escape Studies for Human Monoclonal Antibody AR4A Reveal Isolate-Specific Resistance and a High Barrier to Resistance

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Global control of hepatitis C virus (HCV) depends on development of a prophylactic vaccine. We studied escape for cross-genotype–reactive neutralizing antibody AR4A, providing valuable information for HCV vaccine design. We cultured HCV core-NS2 recombinants H77 (genotype 1a)/JFH1 or the highly antibody-susceptible hypervariable region 1 (HVR1)–deleted variants H77/JFH1$_{\Delta HVR1}$ and J6(genotype 2a)/JFH1$_{\Delta HVR1}$ in Huh7.5 cells with AR4A. Long-term AR4A exposure of H77/JFH1 and H77/JFH1$_{\Delta HVR1}$ did not yield resistance. However, J6/JFH1$_{\Delta HVR1}$ developed the envelope-E2 substitutions I696T or I696N, which reduced AR4A binding (I696N > I696T). I696N conferred greater AR4A resistance than I696T in J6/JFH1$_{\Delta HVR1}$, whereas the reverse was observed in J6/JFH1. This was because I696N but not I696T conferred broadly increased antibody neutralization susceptibility to J6/JFH1. I696N and I696T abrogated infectivity of H77/JFH1 and broadly increased neutralization susceptibility of S52 (genotype 3a)/JFH1. In conclusion, I696 is in the AR4A epitope, which has a high barrier to resistance, thus strengthening the rationale for its inclusion in rational HCV vaccine designs.

Keywords. HCV; HVR1; hypervariable region 1; vaccine; immune evasion; antibody escape; liver disease.

With >71 million individuals chronically infected with hepatitis C virus (HCV) worldwide, approximately 400 000 people die annually from HCV-related liver diseases [1, 2]. Novel direct-acting antiviral (DAA) therapies have cure rates of >95% [2, 3]. However, several factors, including treatment cost, high prevalence of occult infection, low treatment adherence in resource-poor parts of the world, and lack of protection against reinfection underline the necessity of vaccine development [3, 4].

HCV is an enveloped positive-stranded RNA virus of the *Flaviviridae* family, with 6 clinically relevant genotypes [1, 5, 6]. The HCV genome encodes a single polyprotein, which is processed into 3 structural proteins (core, E1, and E2), p7, and nonstructural proteins (NS2–5B). Neutralizing antibodies (NAbs) against HCV target the envelope protein complex E1/E2, which consequently is of primary interest in vaccine development [7, 8].

Studies have shown correlation between NAbs and clearance of acute HCV infection in humans [9, 10]. In addition, passive immunization with NAbs provided partial or even complete protection in chimpanzees [11–13] or human-liver-chimeric mice [14–17]. However, HCV heterogeneity remains a vaccine design obstacle [11]. In addition, HCV rapidly accumulates mutations [18], allowing escape from the humoral immune response. Therefore, successful HCV vaccine development depends on identifying cross-genotype conserved epitopes with high barriers to resistance.

By isolating NAbs from HCV-infected patients, we classified 5 antigenically distinct antigenic regions (termed AR1–5) and showed that AR3-, AR4-, and AR5-specific NAbs are potent in vitro across HCV genotypes, although variation was observed [19, 20]. This variation in cross-genotype susceptibility against AR3–5 antibodies decreased when the E2 motif hypervariable region 1 (HVR1) was deleted, indicating remarkable epitope conservation [21]. Interestingly, the AR4-specific NAb, AR4A, was unique in displaying virtually no cross-genotype variation for HVR1-deleted HCV [21]. Also, AR4A outperformed other promising NAbs, AR3A and AR3B, in protecting a genetically humanized mouse model from HCV challenge [22]. In vivo efficacy of AR4A was confirmed in another mouse challenge study [23]. Finally, AR4A is able to overcome the inherent HCV resistance against neutralization by autologous serum [24] and has high synergy with other potent NAbs [25, 26].

Taking advantage of the high neutralization susceptibility of HVR1-deleted HCV [21, 27, 28], we recently identified high-level AR5A resistance substitutions in cell-culture-infectious, JFH1-based core-NS2 recombinants H77/JFH1 (genotype 1a) and J6/JFH1 (genotype 2a) [29], which suggested that epitope conservation is a poor predictor of the barrier to resistance [29]. When

using the same methods to study AR4A resistance, we were unable to induce escape in H77/JFH1 and observed only low-level resistance in J6/JFH1. Our results indicate that the AR4A epitope has a high barrier to resistance and would be a promising inclusion in the design of future HCV vaccine antigens.

METHODS

Antibodies and Reagents

Human monoclonal antibodies AR3A, AR4A, and AR5A generated from a genotype 1a–infected patient were produced as described previously [19, 20]. 9E10 antibody was kindly provided by Charles Rice [30]. AP33 antibody was kindly provided by Arvind Patel [31]. Plasmids with the core-NS2 sequence from genotypes 1a (H77), 2a (J6), or 3a (S52) and untranslated regions plus the NS3-NS5B region from genotype 2a (JFH1), with or without HVR1, were described previously (S52/JFH1$_{\Delta HVR1}$ has the HVR1-deletion adaptive substitution A369V, and H77/JFH1$_{\Delta HVR1}$ has the HVR1-deletion adaptive substitutions N476D and S733F) [27, 30, 32, 33], as well as a further cell culture–adapted mutant, H77/JFH1$_{\Delta HVR1/N417D/N532D}$, which has 2 additional HVR1-deletion adaptive substitutions, N417D and N532D, in addition to N476D and S733F [29]. J6 and J6$_{\Delta HVR1}$ E1/E2 expression plasmids were previously described [34]. Point mutations were introduced into plasmids by conventional cloning techniques. The HCV sequence of each plasmid was confirmed by sequencing the final DNA preparation (Macrogen).

Cell Culture of Huh7.5 Cells

Huh7.5 cells [35], provided by Charles Rice, were cultured in Dulbecco's modified Eagle's medium (Gibco/Invitrogen) supplemented with 10% of heat-inactivated fetal bovine serum, penicillin 100 U/mL, and streptomycin 100 µg/mL (Gibco/Invitrogen) with 5% CO_2 at 37°C. Cells were split every 48–72 hours.

Transfection of Huh7.5 Cells

Huh7.5 cells were plated at 4×10^5 cells per well in 6-well plates and incubated overnight. HCV RNA was generated by T7-mediated in vitro transcription of XbaI-linearized plasmids. Huh7.5 cells were transfected using Lipofectamine 2000 (Invitrogen). Six hours after transfection, Huh7.5 cells were trypsinized and reseeded into 4 wells of 24-well plates at a cell density of 8×10^4 cells/well, along with plating in 6-well chamber slides for assessing the percentage of infected cells, as described elsewhere [29]. The percentage of infected cells was assessed at the 4 time points, using the primary mouse antibody anti-HCV NS5A 9E10 and the secondary antibody Alexa Fluor 488 goat anti-mouse immunoglobulin G (heavy and light chains; Invitrogen), as previously described [33]. Viral spread was monitored every 24 hours, and supernatants were harvested, sterile filtered, and stored at −80°C. Virus titers were determined as described previously [36, 37].

HCV Escape Assay

Huh7.5 cells were infected with the indicated virus at a multiplicity of infection of 0.001. Virus infection was monitored by immunostaining every 2–3 days as described above. After infection reached 0.1%–1% of the cells, the indicated concentrations of AR4A were added to each well following cell splitting. Cell supernatant was collected and filtered when infection reached 80%–90% of the cells. Direct Sanger sequencing of envelope sequences from cell culture–derived HCV RNA was performed by nested long reverse transcription–polymerase chain reaction procedures, as previously described [29].

Antibody Neutralization

This was done as previously described [21]. We plated 6×10^3 Huh7.5 cells per well in poly-D-lysine 96-well plates and incubated the cells for 24 hours. A virus dose resulting in a final readout of 30–150 focus-forming units of HCV in the control were incubated in quadruplicates with a dilution series of monoclonal antibody and relevant control antibody. Virus-antibody mixes along with 8 replicates of virus only were incubated for 1 hour at 37°C, added to Huh7.5 cells, and incubated for 4 hours at 37°C. Subsequently, the cells were washed, and we added fresh medium and incubated the cells for 48 hours. Cells were fixed and stained with 9E10 antibody, as described previously [33]. The data were normalized with 8 replicates of virus only and were analyzed using 3- or 4-parameter curve fitting in GraphPad Prism.

Immunostaining of HCV-Infected Cells for Evaluating AR4A Binding

Huh7.5 cells were plated at 4×10^5 cells per well in 6-well plates 24 hours before transfection, and transfected with HCV RNA as described above. Twenty-four hours after transfection, 10 000 cells per well were plated in 8-well slides and incubated for 24 hours. Cells were fixed with paraformaldehyde at room temperature for 15 minutes. Anti-E1/E2 antibody (AR4A) [20] and anti-E2 antibody (AP33) [31] were used as primary antibodies at 1 µg/mL with a combination of Alexa Fluor 594 goat anti-mouse immunoglobulin G (heavy and light chains; Invitrogen) and Alexa Fluor 488 goat anti-human (Invitrogen) as secondary antibodies, using a Hoechst (Molecular Probes) nuclear counterstain. Images were acquired using a Zeiss Axio Observer Z1.

Flow Cytometry of AR4A Binding

Huh7.5 cells were transfected with HCV RNA as described above, or 293T cells were transfected with J6 E1/E2 expression plasmids in a similar fashion. A total of 48 hours after transfection, cells were treated with ethylenediaminetetraacetic acid and subjected to 2 phosphate-buffered saline washes between the following steps. Cells were fixed in 4% formaldehyde for 10 minutes and incubated for 1 hour with primary antibodies against E1/E2 (AR4A) [20] and E2 (AP33) [31], both at 1 µg/mL. This was followed by 1-hour incubation with the secondary

antibodies Alexa Fluor 488 anti-human (ThermoFisher) and allophycocyanin anti-mouse (APC; Biolegend). Cells were resuspended and analyzed on a Becton Dickinson LSR Fortessa equipped with 405-, 488-, and 640-nm lasers and suitable filter sets. Data were collected using FACSDiva 8 (Becton Dickinson) and were analyzed with FlowJo 10 software (FlowJo). For data analysis, live cell populations were gated using front- and side-scatter dot plots and were subsequently analyzed using AP33 (APC) and AR4A (Alexa Fluor 488) density plots. The population of double-positive cells was used to calculate mean fluorescence intensity (MFI) and normalized AR4A fluorescence histograms.

RESULTS

Alanine Substitutions That Decrease AR4A Binding to H77 E1/E2 Resulted in Viral Fitness Loss of H77/JFH1

We previously showed that the envelope substitutions P228A, G547A, F550A, H617A, D698A, and Q700A reduced AR4A antibody binding to H77 E1/E2, using alanine scanning [20] (all positions are relative to the H77 reference genome [GenBank accession no. AF009606]). These substitutions were recently shown to have minor effects on the binding of antibodies targeting conformational E2 epitopes, except for F550A and H617A, which both globally destabilized E1/E2 [38]. To identify potentially viable AR4A-resistant viruses, the alanine substitutions were introduced into H77/JFH1 recombinants. Transfecting Huh7.5 cells, we found that P228A greatly reduced fitness, whereas the remaining 5 substitutions resulted in infectivity loss (Figure 1A). A first-passage virus stock was generated for H77/JFH1$_{P228A}$, and the envelope genes were sequenced, revealing the additional substitution M345T (E1; T1374C). In a separate study, we found that H77/JFH1$_{M345T}$ has increased fitness (Velazquez-Moctezuma et al, unpublished data). A virus stock of H77/JFH1$_{M345T}$ without additional envelope substitutions was included when testing AR4A neutralization susceptibility to control for any effect of M345T. Neither P228A nor M345T affected AR4A susceptibility (Figure 1B).

H77/JFH1 and H77/JFH1$_{\Delta HVR1}$ Spread in the Presence of AR4A Without Acquiring Antibody Resistance

To induce AR4A-specific resistance substitutions, we cultured Huh7.5 cells infected with the recombinant virus H77/JFH1 with 200 or 100 µg/mL AR4A (Supplementary Figure 1A and 1B and Table 1). Virus spread in the AR4A-treated culture was significantly delayed. Once the virus spread at day 119 after treatment, we generated a virus stock and sequenced the envelope genes of recovered viruses, revealing the substitutions M345T (E1; T1374C) and D728E (E2; C2525G; Table 1). Testing AR4A susceptibility (Supplementary Figure 1C and Table 1), we found that the treated virus (half-maximal inhibitory concentration [IC$_{50}$], 0.48 µg/mL) and the parental virus (IC$_{50}$, 0.36 µg/mL) were similarly susceptible.
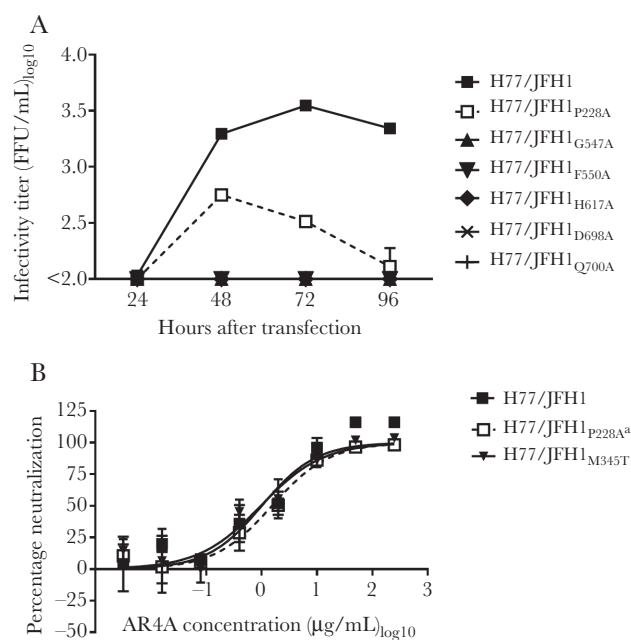

Figure 1. Envelope substitutions shown to reduce AR4A binding at the protein level resulted in highly attenuated viruses. *A*, Huh7.5 cells were transfected with in vitro–transcribed RNA of the indicated H77/JFH1 recombinants. Supernatants were collected at the indicated time points, and HCV infectivity titers were determined. The lower level of quantification was 100 focus-forming units (FFU)/mL. An independent duplicate transfection yielded similar results. *B*, First passages of the indicated viruses were subjected to a dilution series of antibody AR4A, from 250 µg/mL to 0.0032 µg/mL. The virus/antibody mixes, along with virus only, were added to Huh7.5 cells for 4 hours prior to wash and addition of fresh medium. Following 48 hours of infection, the cells were immunostained, and the number of FFU per well was counted as described in Methods. Error bars represent standard error of the mean of 4 replicates normalized to 8 replicates of virus only. The data were analyzed using 3-parameter curve fitting to obtain a sigmoidal dose-response curve. Independent neutralization experiments yielded similar results. ªThe indicated virus had the additional E1 substitution M345T.

Next, we attempted to induce resistant mutants of the HVR1-deleted virus H77/JFH1$_{\Delta HVR1}$, using 5 µg/mL AR4A, in 3 separate cultures (Supplementary Figure 2A and Table 1). Only virus from treatment I spread following 87 days of treatment. In treatments II and III, we were unable to detect infected cells 94 days after treatment. A first-passage treatment I virus stock was generated from supernatant collected at the peak of infection. The envelope sequences had the substitutions T243A (E1; A1068G), L264Q (E1; T1132A), N417D (E2; A1590G), and N532D (E2; A1935G; Table 1). N417D and N532D are previously reported adaptive substitutions for H77/JFH1$_{\Delta HVR1}$ [29]. Testing AR4A susceptibility, we observed that virus from treatment I (IC$_{50}$, 0.011 µg/mL) was similarly susceptible as parental virus (IC$_{50}$, 0.0045 µg/mL; Supplementary Figure 2B and Table 1).

Finally, we cultured a high-fitness HVR1-deleted variant, H77/JFH1$_{\Delta HVR1/N417D/N532D}$ [29], with 20 µg/mL AR4A (Supplementary Figure 2C and 2D). Although the virus spread under AR4A selective pressure, the envelope sequences had not

Table 1. Coding Mutations Observed in the Hepatitis C Virus (HCV) Envelope Proteins Following AR4A Treatment

HCV gene	AR4A concentration	AR4A resistance[c]	Days after treatment[d]	E1	E1	E1	E2	E2	E2	E2	E2	E2	E2	E2	E2	E2	E2
Genome position				1067	1131	1373	1589	1628	1781	1934	1940	1941	2436	2439	2439	2528	2524
H77 abs. ref. (AF009606) position				1068	1132	1374	1590	1629	1782	1935	1935	1936	2425	2428	2428	2517	2525
Nucleotide				A	T	T	A	A	G	A	A	A	A	T	T	T	C
H77/JFH1																	
Treatment I	100 µg/mL[a]	Negligible	119		C												G
H77/JFH1ΔHVR1																	
Treatment I	5 µg/mL	Negligible	87	G	A		G	G	G/A								
H77/JFH1ΔHVR1/N417D/N532D																	
Treatment I	20 µg/mL	ND	130					A/G	G	G	A/G	G					
J6/JFH1ΔHVR1																	
Treatment I	5 µg/mL	Approximately 380	39											C	C		
Treatment II	10 µg/mL	Approximately 880	70										C/A	C			
Treatment III	20 µg/mL	Approximately 25000	83			C									A		
Amino acid number and type																	
Polyprotein position				243	264	345	417	430	481	532	534	534	699	700	700	730	728
H77 abs. ref. (AF009606) position				243	264	345	417	430	481	532	532	532	695	696	696	726	728
Amino acid change				T>A	L>Q	M>T	N>D	N>D	D>N	N>D	N>D	N>S	N>T	I>T	I>N	L>V	D>E
Amino acid observed at least twice in Los Alamos database[b]																	
Within genotypes				TASG	LVMFS	MFVL	NSD	DK	EG	NDTKH	N	N	N	I	I	L	D
Across genotypes (unless also within genotypes)				-	TIY	IA	-	-	NASHMV	-	DTKH	DTKH	G	VTHF	VTHF	-	-

Mutations were identified by direct sequence analysis of HCV RNA amplified by reverse transcription–polymerase chain reaction analysis from viruses recovered from virus passages of the indicated treatments. A single nucleotide for a given position indicates that no other nucleotide was detected, indicating a near-complete shift in the viral population. Two capital nucleotides separated by a dash indicates that both were present in approximately equal proportions. Blue columns denote mutations observed to confer resistance against the neutralizing antibody AR4A.

[a] This virus was first cultured with 200 µg/mL and then passaged onto naive cells, which were cultured with 100 µg/mL.

[b] Data were collected from Los Alamos on 21 August 2017.

[c] The level of AR4A resistance is calculated as the half-maximal concentration for the treated group divided by that of the untreated group.

[d] The numbers represent the total number of days that the virus infection was treated with AR4A before spreading to at least 80% of cells.

acquired novel substitutions. This suggests that the presence of the 2 adaptive substitutions N417D and N532D [29] was sufficient to permit H77/JFH1$_{\Delta HVR1}$ to spread in the presence of AR4A.

Culturing J6/JFH1$_{\Delta HVR1}$ With AR4A Resulted in Escape and High-Level Antibody Resistance

We have previously reported that J6/JFH1 can spread without acquiring resistance, because of high fitness and high resistance to NAbs [29], possibly through a high capacity for cell-to-cell spread as described for SA13/JFH1 [39]. Instead, it is possible to induce J6/JFH1 relevant escape substitutions by culturing J6/JFH1$_{\Delta HVR1}$ with antibodies [29]. Here, we treated cultures with 5, 10, and 20 μg/mL of AR4A (treatments I, II, and III, respectively; Supplementary Figure 3A and Table 1). All displayed significantly delayed spread. Using culture supernatants collected at the peak of infection, we generated first-passage virus stocks and tested AR4A neutralization susceptibility (Supplementary Figure 3B). Viruses from treatment I (IC$_{50}$, 0.065 μg/mL) and treatment II (IC$_{50}$, 0.15 μg/mL) were approximately 600 times more resistant than the parental virus (IC$_{50}$, 0.00017 μg/mL). Virus from treatment III (IC$_{50}$, 4.4 μg/mL) was approximately 40 times more resistant than virus from treatments I and II

and approximately 25 000 times more resistant than untreated J6/JFH1$_{\Delta HVR1}$. Envelope sequencing identified the dominant substitutions N532S (E2; A1936G) and I696T (E2; T2428C) in virus from treatment I, I696T (E2; T2428C) in virus from treatment II, and I696N (E2; T2428A) and L726V (E2; T2517G; Table 1) in virus from treatment III. This implicated position I696 in AR4A resistance.

Substitutions at Position I696 Conferred AR4A Resistance to J6/JFH1$_{\Delta HVR1}$

Substitutions I696T, I696N, and L726V or the combination I696N/L726V were introduced into J6/JFH1$_{\Delta HVR1}$. Transfecting Huh7.5 cells with these recombinants, we found that I696N greatly decreased virus fitness. L726V increased fitness, and virus with the combination I696N/L726V had titers slightly higher than J6/JFH1$_{\Delta HVR1}$ (Figure 2A). Virus titers were unaffected by I696T. We generated first-passage stocks of all 4 viruses and determined their envelope sequences. Only J6/JFH1$_{\Delta HVR1/I696T}$ acquired the substitution, A217G (E1; C994G). Testing AR4A neutralization susceptibility, we found that J6/JFH1$_{\Delta HVR1/L726V}$ (IC$_{50}$, 0.000016 μg/mL) was similarly susceptible, compared with J6/JFH1$_{\Delta HVR1}$ (IC$_{50}$, 0.000026 μg/mL). J6/JFH1$_{\Delta HVR1/I696N}$ (IC$_{50}$, 0.62 μg/mL) and J6/JFH1$_{\Delta HVR1/I696N/L726V}$ (IC$_{50}$, 0.41 μg/mL) were approximately 10 000 less susceptible to AR4A. Finally,
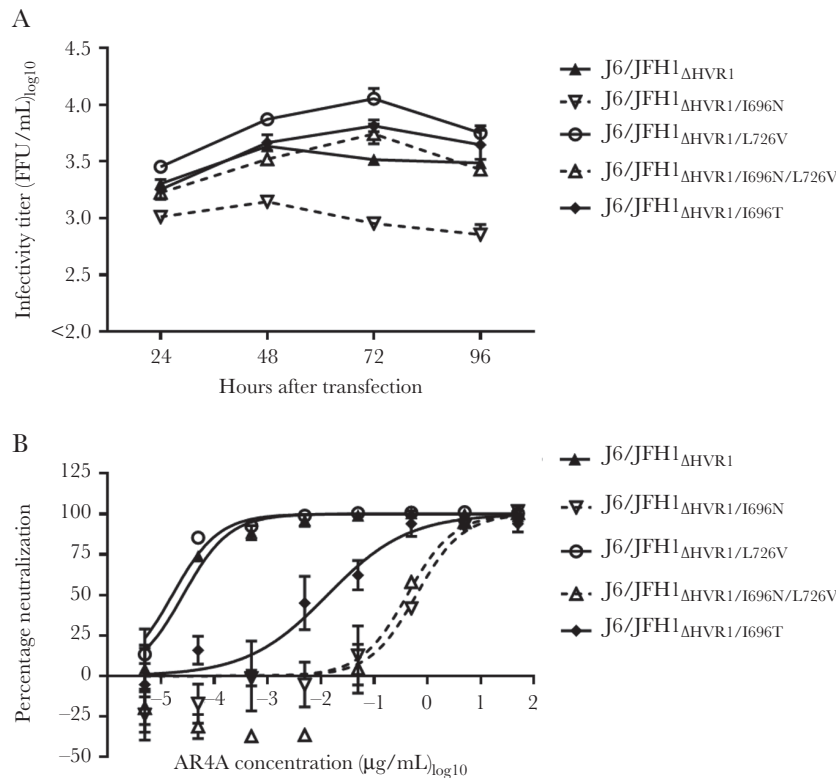

Figure 2. The substitutions I696T and I696N conferred AR4A resistance to J6/JFH1$_{\Delta HVR1}$. *A*, Huh7.5 cells were transfected with in vitro–transcribed RNA of the indicated recombinants harboring I696T, I696N, L726V or the combination I696N/L726V. Error bars represent standard errors of the mean (SEMs). Supernatants were collected at the indicated time points, and hepatitis C virus infectivity titers were determined. Lower level of quantification was 100 focus-forming units (FFU)/mL. *B*, First passages of the viruses were subjected to a dilution series of AR4A, from 50 μg/mL to 0.000005 μg/mL, and neutralization was assessed and analyzed as described in Figure 1, except we used 4-parameter curve fitting to obtain a sigmoidal dose-response curve. Error bars represent SEMs. Independent neutralization experiments yielded similar results.
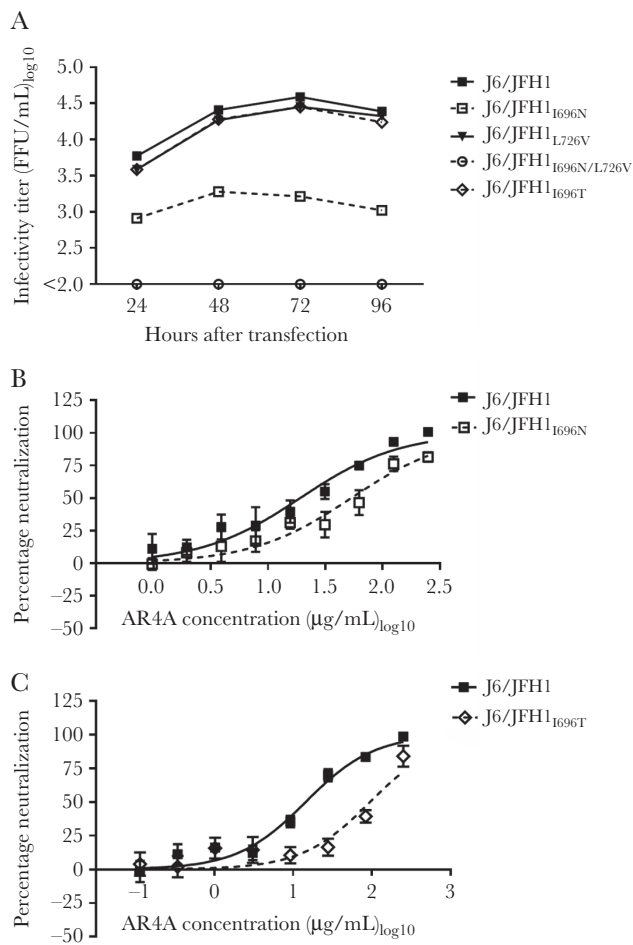
Figure 3. The substitutions I696N and I696T conferred AR4A resistance to the parental recombinant virus J6/JFH1. *A*, Huh7.5 cells were transfected with in vitro–transcribed RNA of the indicated recombinants with single or combined mutations. Supernatants were collected at the indicated time points, and hepatitis C virus infectivity titers were determined. The lower level of quantification was 100 focus-forming units (FFU)/mL. *B* and *C*, First passages of the indicated viruses were subjected to a dilution series of AR4A, from 250 µg/mL to 0.9 µg/mL (*B*) or from 250 µg/mL to 0.1 µg/mL (*C*), and neutralization was assessed and analyzed as described in Figure 1, except we used 4-parameter curve fitting to obtain a sigmoidal dose-response curve. Error bars represent standard errors of the mean. Independent neutralization experiments yielded similar results.

J6/JFH1$_{\Delta HVR1/I696T}$ (IC$_{50}$, 0.011 µg/mL) was approximately 420 times less susceptible, compared with J6/JFH1$_{\Delta HVR1}$ (Figure 2B). Thus, we showed that I696N conferred high-level AR4A resistance to J6/JFH1$_{\Delta HVR1}$, while L726V compensated virus fitness loss. I696T conferred slightly lower AR4A antibody resistance, compared with I696N, but did not affect virus fitness.

I696T Conferred Higher AR4A Resistance Than I696N for J6/JFH1 Retaining HVR1

Substitutions I696T, I696N, and L726V or I696N/L726V were introduced into J6/JFH1. Transfecting Huh7.5 cells, we observed that in contrast to J6/JFH1$_{\Delta HVR1}$, virus titers were not measurable for J6/JFH1$_{I696N/L726V}$. I696N alone greatly decreased virus viability, while I696T did not (Figure 3A).

We generated first-passage stocks and confirmed the envelope sequences. Testing AR4A neutralization susceptibility, we found that J6/JFH1$_{I696N}$ (IC$_{50}$, 53 µg/mL) was approximately 3 times less susceptible, compared with J6/JFH1 (IC$_{50}$, 19 µg/mL; Figure 3B). J6/JFH1$_{I696T}$ (IC$_{50}$, 95 µg/mL) was approximately 7 times less susceptible, compared with J6/JFH1 (IC$_{50}$, 14 µg/mL; Figure 3C).

AR4A Binding to J6 E1/E2 Was Reduced More by I696N Than by I696T

To assess the effects of I696T and I696N on AR4A binding to E1/E2, we performed immunostaining of Huh7.5 cells transfected with J6/JFH1$_{\Delta HVR1}$, J6/JFH1$_{\Delta HVR1/I696T}$, or J6/JFH1$_{\Delta HVR1/I696N}$ recombinants, using AR4A and the control E2 antibody, AP33 (Figure 4A). AP33 signal appeared unaltered, indicating similar levels of E2. I696N had a decreased AR4A signal, compared with the parental virus, whereas I696T had no or marginal effect. Immunostaining of Huh7.5 cells transfected with J6/JFH1, J6/JFH1$_{I696T}$, and J6/JFH1$_{I696N}$ confirmed these findings in the corresponding viruses with HVR1 (Figure 4B).

To better quantify the effects of I696T and I696N on AR4A binding, we performed flow cytometry of Huh7.5 cells transfected with these same recombinants (Figure 4C; one of 2 independent experiments shown in Supplementary Figure 4). I696T reduced AR4A binding less than I696N both in the absence and the presence of HVR1. To assess AR4A binding to E1/E2 in the absence of other HCV proteins, we transfected 293T cells with the analogous E1/E2 expression vectors (Figure 4D; one of 2 independent experiments shown in Supplementary Figure 5), which confirmed the finding.

I696N Conferred Broadly Increased Neutralization Susceptibility in an HVR1-Dependent Manner, Whereas I696T Did Not

To investigate how I696T conferred higher AR4A resistance than I696N to HCV retaining HVR1, we tested the neutralization susceptibility of J6/JFH1 and J6/JFH1$_{\Delta HVR1}$ recombinants with these substitutions, using AR3A and AR5A [19, 20]. J6/JFH1$_{I696N}$ (IC$_{50}$, 0.028 µg/mL) was approximately 1300 times more susceptible to AR3A, compared with J6/JFH1 (IC$_{50}$, 38 µg/mL; Figure 5A), and approximately 90 times more susceptible to AR5A (IC$_{50}$, 0.22 µg/mL), compared with J6/JFH1 (IC$_{50}$, 20 µg/mL; Figure 5B). In the absence of HVR1, I696N did not increase HCV susceptibility to neutralization (Figure 5A and 5B). The substitution I696T did not significantly affect neutralization susceptibility in J6/JFH1, with or without HVR1 (Figure 5C and 5D). This indicated that I696N broadly increased neutralization susceptibility in an HVR1-dependent manner, whereas I696T did not.

Substitutions at Position I696 Affected Fitness and Antibody Susceptibility in Genotypes 1a and 3a

We introduced I696T and I696N in core-NS2 recombinants of genotype 1a (H77/JFH1) and 3a (S52/JFH1), with and without HVR1, and transfected Huh7.5 cells. I696T and I696N greatly
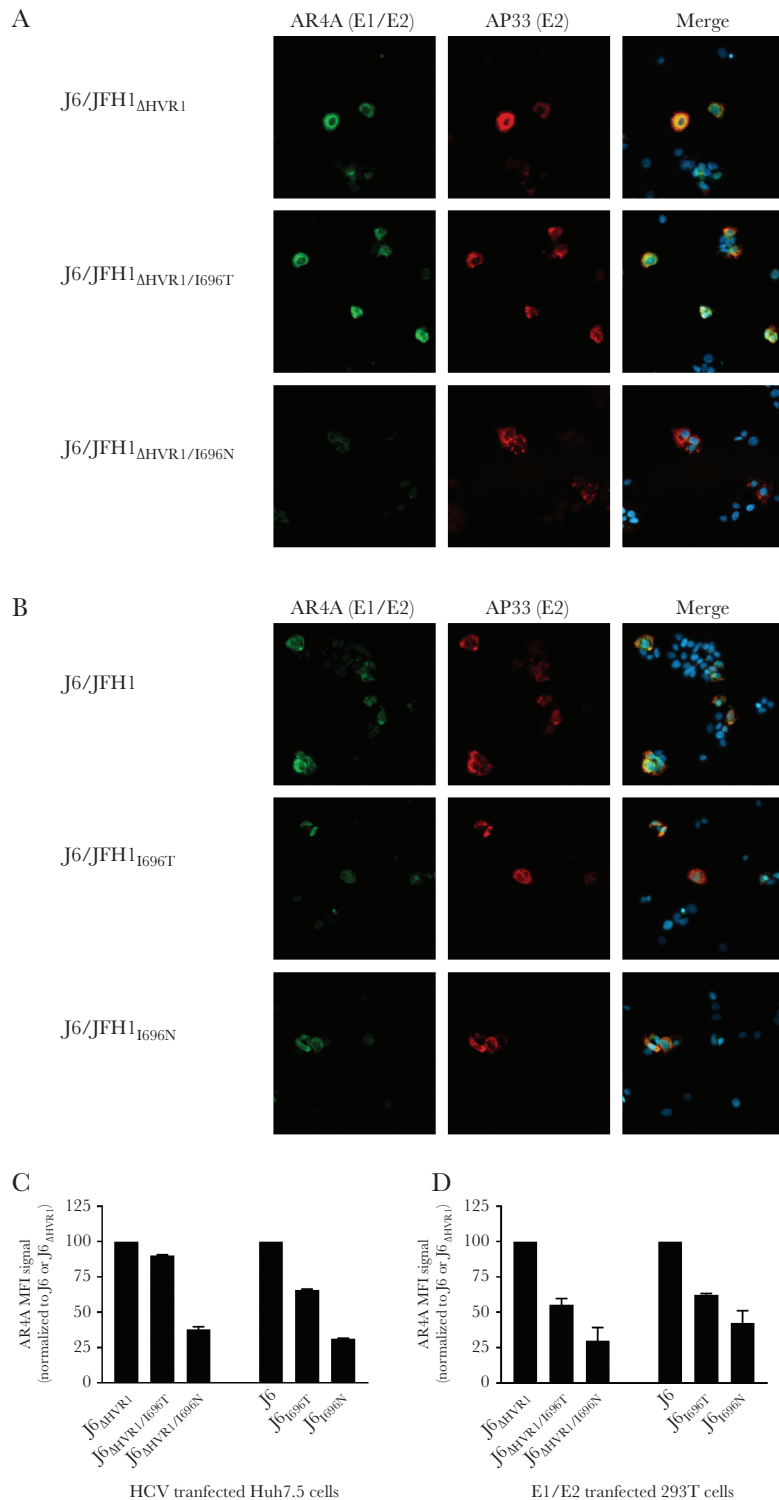
Figure 4. AR4A binding to HCV J6 E1/E2 was reduced more by I696N than by I696T. *A,* Huh7.5 cells were transfected with in vitro–transcribed RNA of the indicated J6/JFH1$_{\Delta HVR1}$ recombinants. Cells were fixed 48 hours after transfection and stained using the primary antibodies AR4A (anti-E1/E2) and AP33 (anti-E2) as described in Methods. Cell nuclei were visualized with Hoechst. *B,* Huh7.5 cells were transfected with in vitro–transcribed RNA of the indicated J6/JFH1 recombinants and stained as described above. *C,* Huh7.5 cells were transfected with in vitro–transcribed RNA of the indicated J6/JFH1 recombinants with and without HVR1. Cells were prepared for flow cytometry by releasing them into suspension, fixing them, and staining them using the primary antibodies AR4A (anti-E1/E2) and AP33 (anti-E2) as described in Methods. The AR4A mean fluorescence intensity (MFI) was calculated for the double-positive cell population as shown in Supplementary Figure 4 and normalized to either J6 or J6$_{\Delta HVR1}$, as appropriate. The bars represent the means (±SDs) of the 2 independent experiments. *D,* 293T cells were transfected with the indicated J6 E1/E2 expression vectors with and without HVR1. Cells were prepared for flow cytometry by releasing them into suspension, fixing them, and staining them using the primary antibodies AR4A (anti-E1/E2) and AP33 (anti-E2) as described in Methods. The AR4A MFI was calculated for the high-AR4A double-positive cell population as shown in Supplementary Figure 5 and normalized to either J6 or J6$_{\Delta HVR1}$, as appropriate. The bars represent the means (±SDs) of the 2 independent experiments.
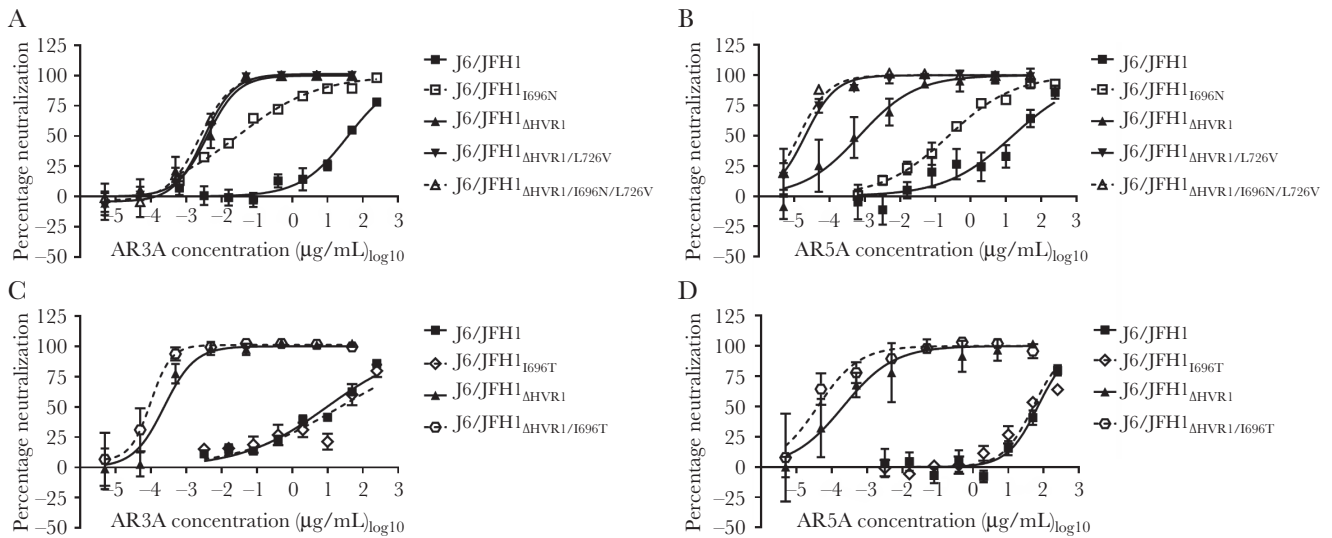
Figure 5. I696N greatly increased the antibody susceptibility of J6/JFH1 retaining HVR1 against the neutralizing antibodies AR3A and AR5A, whereas I696T did not. First passages of the indicated viruses were subjected to a dilution series of antibodies AR3A (*A* and *C*) or AR5A (*B* and *D*), and neutralization was assessed and analyzed as described in Figure 1, except we used 4-parameter curve fitting to obtain a sigmoidal dose-response curve. Error bars represent standard errors of the mean. Independent neutralization experiments yielded similar results.

decreased the fitness of both H77/JFH1 and H77/JFH1$_{\Delta HVR1}$ (Supplementary Figure 6). This was also the case for S52/JFH1$_{\Delta HVR1}$ (Figure 6A). For S52/JFH1, I696N greatly decreased

the fitness, while I696T did not (Figure 6A). We generated first-passage virus stocks of S52/JFH1$_{I696T}$ and S52/JFH1$_{I696N}$. Sequencing revealed that S52/JFH1$_{I696N}$ had acquired the
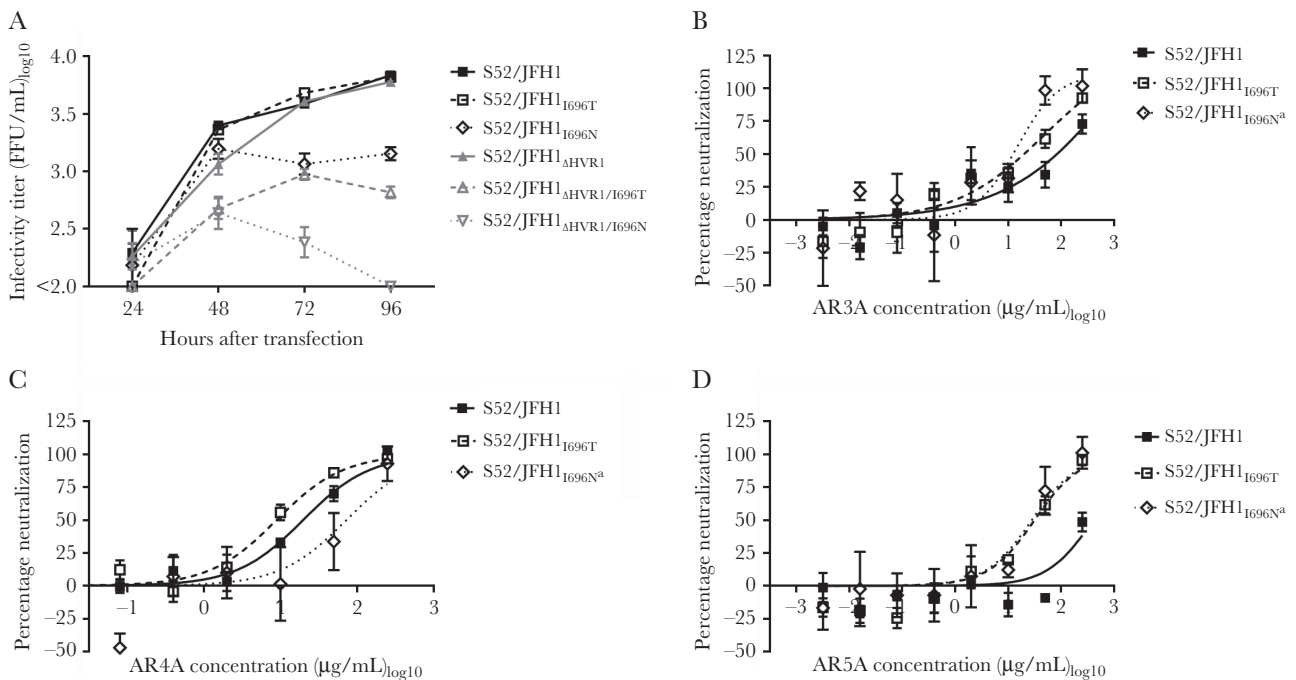

Figure 6. Substitutions at position I696 had varied effects on virus fitness and antibody susceptibility of hepatitis C virus (HCV) genotype 3a recombinants. *A*, Huh7.5 cells were transfected with in vitro–transcribed RNA of the indicated S52/JFH1 recombinants (genotype 3a). Supernatants were collected at the indicated time points, and HCV infectivity titers were determined. The lower level of quantification was 100 focus-forming units (FFU)/mL. *B–D*, First passages of the indicated viruses were subjected to a dilution series of AR3A (*B*), AR4A (*C*), and AR5A (*D*), from 250 µg/mL to 0.0032 µg/mL, and neutralization was assessed and analyzed as described in Figure 1, except we used 4-parameter curve fitting to obtain a sigmoidal dose-response curve. Error bars represent standard errors of the mean. Independent neutralization experiments yielded similar results. [a]The indicated virus had the additional E2 substitution V553A.

substitution V553A (E2; C1999T), whereas S52/JFH1$_{I696T}$ did not have additional substitutions. Next, we tested neutralization susceptibility against AR3A (Figure 6B), AR4A (Figure 6C), and AR5A (Figure 6D). While both I696T and I696N increased susceptibility against AR3A and AR5A, we observed an approximately 4-fold increase of AR4A-specific resistance for S52/JFH1$_{I696N}$.

DISCUSSION

AR4A targets a conserved epitope and has a superior protective effect in animal models [20, 21, 23]. Here, we used a newly established method to study AR4A escape and found that tested isolates were incapable of developing high-level AR4A resistance. In addition, we showed that position I696 is in the AR4A epitope. In addition, substitutions at this position were found to have isolate-dependent effects, including potential fitness loss and broadly increased neutralization susceptibility.

We analyzed alanine substitutions that we previously showed to have decreased AR4A binding to H77 E1/E2 [19, 20]. Infectivity of H77/JFH1 was abrogated except for P228A, which did not confer AR4A resistance. Although additional alanine substitutions recently reported to affect AR4A binding [38] could result in resistant viable viruses, the low infectivity of the alanine mutants tested here suggests that in vitro escape studies represent a more tenable approach to identify viable resistance substitutions.

Several studies have demonstrated a correlation between in vitro resistance substitutions and resistance substitutions found in vivo [40–44]. H77/JFH1 did not develop AR4A-specific resistance substitutions, even after long-term high-dose antibody culture. The virus developed the E1 substitution M345T that we have shown is adaptive, suggesting the virus evaded neutralization through improved fitness, as previously described for J6/JFH1 cultured with AR5A [29]. Similarly, upon culturing H77/JFH1$_{\Delta HVR1}$ in the presence of AR4A, we identified the substitutions N417D and N532D, which abolish E2 glycosylation sites N417 and N532 and have been implicated in increasing antibody susceptibility and virus fitness [45]. We have previously shown that N417 did not affect AR5A susceptibility for HVR1-deleted virus [29] and that N417D together with N532D greatly increased the fitness of H77/JFH1$_{\Delta HVR1}$ [29]. When culturing H77/JFH1$_{\Delta HVR1/N417D/N532D}$ with AR4A, the virus spread without acquiring additional envelope substitutions. These results indicate that the increased fitness of H77/JFH1 and H77/JFH1$_{\Delta HVR1}$ permitted virus spread by rendering the antibody dose too low to prevent spread. We previously reported that, with comparable antibody doses, both H77/JFH1$_{\Delta HVR1}$ and J6/JFH1$_{\Delta HVR1}$ developed resistance substitutions against AR5A after <30 days of culture [29]. The fact that H77/JFH1 and H77/JFH1$_{\Delta HVR1}$ took 87–130 days to spread with AR4A (only acquiring increased fitness) supports that it is difficult for H77/JFH1 to develop AR4A-specific resistance substitutions.

Culturing J6/JFH1$_{\Delta HVR1}$ in the presence of AR4A for 39–83 days, we identified substitutions at position I696 that increased AR4A resistance for J6/JFH1, with and without HVR1. This position is completely conserved within genotype 2 and 93% conserved across genotypes with 2.4% threonine (Los Alamos HCV sequence database). I696N greatly decreased AR4A neutralization susceptibility for J6/JFH1$_{\Delta HVR1}$ but required the fitness-compensating substitution L726V, which decreased the fitness of J6/JFH1. I696T had a lower effect on AR4A binding than I696N, as shown by immunofluorescence and flow cytometry. This was reflected in the fact that I696T conferred less resistance than I696N against AR4A for J6/JFH1$_{\Delta HVR1}$. Interestingly, I696T conferred greater resistance than I696N for J6/JFH1, likely because I696N broadly increased neutralization susceptibility for J6/JFH1 but not for J6/JFH1$_{\Delta HVR1}$. I696T did not affect the fitness of J6/JFH1 with or without HVR1 and did not affect susceptibility to other NAbs for either virus, thus indicating that it is a de novo AR4A-specific resistance substitution.

In a previous complete alanine scanning mutagenesis study of recombinant E1/E2, alanine substitution at I696 had no effect on AR4A binding, whereas D698A and Q700A substitutions reduced AR4A binding [20, 38]. Combined, these results firmly establish that E2 positions 696–700 form part of the AR4A epitope, although AR4A co-crystallization of E1/E2 would be needed to fully elucidate the epitope. Our data complement recent findings of polymorphisms in E1/E2 that have been shown to broadly modulate NAb susceptibility, including susceptibility to AR4A [39, 46, 47].

Interestingly, we found that H77/JFH1 was nonviable when I696T or I696N were introduced. For S52/JFH1, we observed some effect on the viability of I696N but not of I696T. I696N decreased AR4A susceptibility, but both I696T and I696N broadly increased NAb susceptibility. Thus, our data indicate that substitutions at position I696 can have isolate-dependent effects on virus fitness and broad NAb susceptibility.

We previously reported that substitutions of L665 conferred high-level AR5A resistance in the 6 major HCV genotypes [29]. Here, we found that I696T conferred low-level AR4A resistance to J6/JFH1. Together with our inability to induce resistance in H77/JFH1 despite long-term cultures with antibody, this indicates that the barrier to resistance for AR4A is higher than that for AR5A. While the promise of epitope-targeted vaccine development is still being explored [48], 2 studies have shown some efficacy for linear HCV envelope peptides [49, 50]. However, these approaches cannot be readily adapted to NAbs targeting nonlinear conformational epitopes, such as AR4A. A better approach might be to emphasize the AR4A epitope in E1/E2 vaccine antigens, but improved structural understanding of the E1/E2 heterodimer is needed.

In conclusion, by culturing viruses in the presence of the vaccine-relevant NAb AR4A, we identified the resistance

substitution I696T for J6/JFH1, whereas no resistance could be induced for H77/JFH1. Our findings indicate that AR4A targets a low-variation epitope with a high barrier to resistance, which in conjunction with high AR4A cross-genotype conservation makes the epitope a prime candidate for inclusion in HCV vaccine candidates.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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