Highly Efficient JFH1-Based Cell-Culture System for Hepatitis C Virus Genotype 5a: Failure of Homologous Neutralizing-Antibody Treatment to Control Infection

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Background. Recently, a hepatitis C virus (HCV) cell-culture system was developed that employed strain JFH1 (genotype 2a), and JFH1-based intra- and intergenotypic recombinants now permit functional studies of the structural genes (Core, E1, and E2), p7, and NS2 of genotypes 1–4. The goal was to adapt the system to employ genotype 5.

Methods. Huh7.5 cells infected with SA13/JFH1, containing Core-NS2 of strain SA13 (genotype 5a), were monitored for Core expression and for supernatant infectivity and HCV-RNA titers. Adaptive mutations of SA13/JFH1 were identified by sequence analysis of recovered genomes and reverse-genetic studies. Receptor blockage was performed with anti-CD81 and anti–SR-BI. For neutralization experiments, SA13/JFH1 or JFH1-based viruses of other genotypes were incubated with patient sera.

Results. SA13/JFH1 with NS2 and NS3 mutations yielded infectivity titers >10^5 TCID50/mL. Infection with SA13/JFH1 was inhibited by CD81 blocking and SR-BI blocking, respectively, and by preincubation with genotype 5a chronic-phase patient sera. Such sera had varying cross-genotype neutralization potential. However, preincubation and treatment with homologous neutralizing antibodies could not control SA13/JFH1 infection in culture.

Conclusion. The SA13/JFH1 culture permits genotype 5a–specific studies of Core–NS2 function and interfering agents. The ability of HCV to spread in vivo during treatment with neutralizing antibodies was confirmed in vitro.

Hepatitis C virus (HCV), which infects ~180 million people worldwide, is a leading cause of end-stage liver disease and liver transplantation. No vaccine is available, and therapy with interferon and ribavirin cures only ~50% of treated patients, depending on the genotype [1].

In vitro studies recapitulating the complete HCV life cycle in the human hepatoma cell line Huh7 became possible after identification of the genotype 2a isolate JFH1 and the improved J6/JFH (2a/2a) recombinant [2–4]. Furthermore, JFH1-based cultures have been developed for genotypes 1a, 1b, 3a, and 4a [5–8]. In these intergenotypic recombinants, the structural genes (Core, E1, and E2), p7, and NS2 of JFH1 were replaced by genotype-specific sequences, and efficient growth depended on various adaptive mutations. However, no such systems were available for genotypes 5 and 6.

Genotype 5a was originally identified in a cohort of South African patients with HCV-induced hepatocellular carcinoma [9, 10]; this genotype accounts for >30% of HCV infections in South Africa [11]. In addition, it has been reported to have spread into Europe, with local prevalences of up to 30% [12, 13]. Little is known about the virology, clinical features, and treatment susceptibil-
ity of this particular genotype, but there are indications that, like genotypes 2 and 3, it might have a favorable response to treatment [14, 15].

To broaden genotype-specific studies, including studies of neutralizing antibodies, studies that are thought to be an important prerequisite of vaccine design, we developed a JFH1-based intergenotypic recombinant genotype 5a cell-culture system. We identified adaptive mutations and demonstrated the applicability of 5a/JFH1 viruses for specific entry studies, including infection by blocking of the CD81 and SR-BI coreceptors on cells and by preincubation of the virus with sera from patients infected with autologous (same strain as in recombinant 5a/JFH1) and homologous (different 5a strains) genotype 5a isolates. Finally, we determined the cross-genotype neutralization potential and the in vitro therapeutic potential of genotype 5a chronic-phase sera.

MATERIALS AND METHODS

Construction of pSA13/JFH1. The Core–NS2 sequence of strain SA13 (genotype 5a) was constructed by fusion polymerase chain reaction (PCR) from pCMV-SA13 and clonal sequences recovered from plasma of a chimpanzee infected with serum from a South African patient with hepatocellular carcinoma [16, 17]. The JFH1–SA13 junctions at Core and NS2 were made by fusing this fragment to amplicons obtained from pJFH1 (provided by T. Wakita, Tokyo Metropolitan Institute for Neuroscience) [2]. An XmnI/SpeI fragment of the final fusion product was introduced into pFL-J6/JFH (provided by C. Rice, Rockefeller University) [3] to generate pSA13/JFH1 (GenBank accession no. FJ393024). The SA13 sequence of pSA13/JFH1 deviated from the consensus sequence at 2 positions (C973T and C910G), resulting in amino acid change N384S in hypervariable region 1 (HVR1) of E2. Replication-deficient pSA13/JFH1-GND was created by insertion of the XmnI/SpeI fragment of pSA13/JFH1 into pFL-J6/JFH (GND) [3]. Point mutations in pSA13/JFH1 were inserted by fusion PCR and cloning. All PCRs were performed with Pfu polymerase (Stratagene). The HCV sequences of plasmids were verified by sequencing of the final DNA preparation (EndoFree Plasmid Maxi Kit; Qiagen).

Transfection and infection of Huh7.5 cells. Huh7.5 cells were cultured as described elsewhere [7]. At ~24 h before transfection or infection, ~4 × 10^5 Huh7.5 cells/well were seeded into a 6-well plate (Nunc). Plasmids were digested by use of XbaI (New England Biolabs) and were treated with mung-bean nuclease (New England Biolabs) [8]. RNA was transcribed by T7 RNA polymerase (Promega), for 2 h at 37°C. Transfection was performed with 5 μL of Lipofectamine 2000 (Invitrogen) and 2.5 μg of RNA transcript, in serum-free medium (Opti-MEM; Invitrogen). On the basis of the percentage of Core-positive cells on day 1, it appeared that transfection efficiency was similar for the different constructs in each experiment. For infection, cells were incubated with 1 mL of cell-free supernatant for 4–5 h, unless stated otherwise.

Monitoring of HCV-infected cell cultures. Huh7.5 cells were immunostained for HCV Core, with mouse anti–HCV Core protein monoclonal antibody (B2) (Anogen; Yes Biotech Laboratories) and with secondary antibody, Alexa Fluor 594–conjugated goat anti-mouse IgG (H+L) (Invitrogen); cell nuclei were counterstained with Hoechst 33342 (Molecular Probes) [7].

In culture supernatants, HCV infectivity titers were determined by the TCID50 method, and HCV RNA titers were measured by TaqMan real-time PCR assay, with a detection limit of 500 IU/mL, as described elsewhere [7].

Sequence analysis of cell culture–derived SA13/JFH1 viruses. Direct sequence analysis of recovered viral genomes was performed as described elsewhere [7], with the exception of second-round PCR primer mixes for amplicons 1–5, which were as follows: amplicon 1, 1–845_HCV-MOD (GTAGCGTGGTGTTGGAGAACCCCTCGTGAT) and SA13.1seqR1443 (CAGGATGGCAGGCCGTAGTATG); amplicon 2, ChimseqF809 (GGATCTCTAGGAGGATGGAATCTATGCTGAT) and SA13JFH1R1987 (CTTGAAAACCCCTGGAAATTTCACAGGTCGCCACCAAACAG); amplicon 3, SA13JFH1F1827 (GAAATGGGTAGCCAGCATGTTCTCTC) and SA13R2525 (CAGCAAGTTAAGAGGCAGCCGACTATG); amplicon 4, SA13.2seqF2327 (CTGAGCGCGCTCTCATACACAC) and SA13R3432 (CTGTGAGGCAGCCATGTTCTC) and SA13R3432 (CTGTGAGGCAGCCATGTTCTC) and SA13R3432 (CTGTGAGGCAGCCATGTTCTC) and SA13R3432 (CTGTGAGGCAGCCATGTTCTC) and 4118R_JFH1 (CGCCCGAGGCCGCTCTCTCTCTCTCTCT). For clonal analysis, a long (nucleotide positions [np] 86–7541) reverse-transcriptase PCR product was amplified by use of primers ~2855_HCV-MOD and 7234R_JFH1, as described elsewhere [7], and was cloned into PCR-XL-TOPO (Invitrogen); multiple clones were sequenced and analyzed [7].

Analysis of entry of SA13/JFH1, by CD81 and SR-BI blocking and incubation with genotype 5a patient sera or purified IgG. At ~24 h before the assays, ~6 × 10^5 Huh7.5 cells/well were plated on poly-L-lysine–coated 96-well plates (Nunc). For CD81 blocking, cells were incubated with either anti-CD81 (JS-81; BD Biosciences, Pharmingen) or isotype-matched control antibody (anti–human immunodeficiency virus, p24, clone Kal-1; DAKO) [7, 8]. For SR-BI blocking, cells were first incubated for 1 h with either rabbit polyclonal anti-SR-BI (GeneTex) or rabbit polyclonal control antibody (anti–human retinoblastoma Ab-6; Thermo Scientific), ~150 focus-forming units (FFUs) of either SA13/JFH1 or J6/JFH were added, and cells were then incubated for 3 h before being washed once with PBS. Experiments were performed in triplicate, unless stated otherwise. Cells were incubated for 2 days in complete medium and then were stained to determine the number of FFUs, as described elsewhere [8]. The percentage of inhibition...
by anti-CD81 and anti–SR-BI was calculated by comparison with the mean FFUs of 3 replicate wells incubated with virus only.

For neutralization assays, heat-inactivated (30 min at 56°C) sera were preincubated, for 1 h at 37°C, either with 50, 100, 200, 400, or 800 TCID$_{50}$'s of SA13/JFH1 or J6/JFH (from day 3 of a first-passage experiment, derived from transfections shown in panel A). Mean ± SD supernatant infectivity titers (left y-axis) and supernatant hepatitis C virus (HCV) RNA titers (right y-axis) were determined in duplicate by a TCID$_{50}$ assay and quantitative HCV TaqMan real-time polymerase chain reaction assay, respectively. An asterisk (*) indicates an infectivity titer <10$^1$ TCID$_{50}$/mL (<3 of 6 replicate wells infected by undiluted supernatant) in 2 independent determinations; a pound sign (#) indicates infectivity titers 10$^1.3$ and 10$^1$ TCID$_{50}$/mL, respectively, in 2 independent determinations.

Neutralization experiments with patient sera and IgG were performed in triplicate, and, after a 3-h incubation, cells were washed with PBS and were incubated with complete medium for 2 days and then were stained to determine the number of FFUs [8]. The percentage of inhibition by patient sera was calculated by comparing the latter with the serum control [8]. We have found that, when 1:50 or 1:100 dilutions of the serum control are incubated with each genotype of the virus, the resulting FFUs are similar to those for the corresponding virus-only control (data not shown). Thus the percentage of inhibition by purified IgG was calculated by comparison with 6 replicates of the virus-only control. Genotype 5a chronic-phase sera (SA1, SA3, and SA13) used for neutralization were from South African patients with hepatocellular carcinoma [9, 18].

HCV-infected cells: treatment with neutralizing antibodies. A 1:50 or 1:200 dilution of heat-inactivated patient serum (SA3) was preincubated with ~50 or ~100 TCID$_{50}$'s of SA13/JFH1 virus, respectively, for 1 h at 37°C, and was added to ~1 × 10$^5$ or ~1.5 × 10$^6$ Huh7.5 cells/well, respectively, of a 24-well plate (Nunc) for 24 h. Cells were washed with PBS, and fresh medium supplemented with SA3 serum, at dilutions of 1:50 or 1:200, respectively, was added every 24 h. Both experiments were performed in parallel with heat-inactivated serum control under the same conditions. The envelope sequences of

**Figure 1.** Viability of SA13/JFH1 in Huh7.5 cells. A and B, Two independent transfections of Huh7.5 cells, with RNA transcript of either pSA13/JFH1 or pJ6/JFH. Mean ± SD supernatant infectivity titers were determined in duplicate by a TCID$_{50}$ assay (left y-axis). C, Comparison of growth kinetics of passaged SA13/JFH1 and J6/JFH viruses in Huh7.5 cells. Huh7.5 cells were incubulated with sterile filtered supernatants containing ~10$^3$ TCID$_{50}$ of either SA13/JFH1 or J6/JFH (from day 3 of a first-passage experiment, derived from transfections shown in panel A). Mean ± SD supernatant infectivity titers (left y-axis) and supernatant hepatitis C virus (HCV) RNA titers (right y-axis) were determined in duplicate by a TCID$_{50}$ assay and quantitative HCV TaqMan real-time polymerase chain reaction assay, respectively. An asterisk (*) indicates an infectivity titer <10$^1$ TCID$_{50}$/mL (<3 of 6 replicate wells infected by undiluted supernatant) in 2 independent determinations; a pound sign (#) indicates infectivity titers 10$^1.3$ and 10$^1$ TCID$_{50}$/mL, respectively, in 2 independent determinations.
recovered viruses were determined on the basis of amplicons obtained from the supernatant in a JFH1-specific reverse-transcriptase PCR with primer 4118R_JFH1 (see above) and PCR primers /H11002 285S_HCV-MOD [7] and JFH1R4141 (GGATTGATGCCATGTGCCTTGGATAGGTAC). In both treatment experiments, amplicons for use in direct sequencing were generated by use of primer mixes for amplicons 1–4 (see above). For clonal analysis of the amplicons from the low-dose (1:200-dilution) experiment, the PCR product (np 86–4140) was cloned into pCR-XL-TOPO (Invitrogen) and was sequenced to further analyze a position for which direct sequencing showed evidence of change (see the Results section).

RESULTS

Development and cell-culture adaptation of 5a/2a intergenotypic recombinant. The recombinant SA13/JFH1 was generated in a manner analogous to that used to generate the JFH1-based recombinants of genotypes 1–4 [3, 5–8]. Thus, SA13/JFH1 contains the complete 5' untranslated region (UTR) of JFH1 (np 1–340; nucleotide and deduced amino acid positions refer to the SA13/JFH1 sequence), the structural genes (Core, E1, and E2), p7, and NS2 of the genotype 5a isolate SA13 (np 341–3421), and NS3-NS5B, as well as the 3' UTR of JFH1 (np 3422–9669).

After 2 independent transfections of Huh7.5 cells with RNA transcripts, we found that SA13/JFH1 yielded supernatant infectivity titers of $\times 10^{1.3}$ TCID$_{50}$/mL for the first 6 days after transfection (figure 1A and 1B). However, the J6/JFH-positive control culture, showing infectivity titers $\times 10^8$ TCID$_{50}$/mL on day 1 (figure 1B), yielded titers of $10^5$–$10^6$ TCID$_{50}$/mL on day 6 (figure 1A and 1B), when HCV Core was expressed in most cells of the culture. For SA13/JFH1, similar infectivity titers and a high percentage of infected cells were not observed until day 13 and day 10, respectively (figure 1A and 1B). SA13/JFH1-GND cultures remained Core negative throughout the follow-up period.

During serial passages of SA13/JFH1 viruses from culture supernatants from one of the transfection experiments, we ob-

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**Table 1. Nucleotide changes revealed by direct sequencing of SA13/JFH1 viruses recovered from cell-culture supernatants.**

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<th>2728</th>
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<th>3696</th>
<th>4277</th>
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<td>pSA13/JFH1</td>
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<td>C</td>
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<td>Transfection experiment with original SA13/JFH1b</td>
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<td>•</td>
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<td>Day 7,d</td>
<td>•</td>
<td>G/t</td>
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<td>Day 8</td>
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<td>Transfection experiment with mutated SA13/JFH1</td>
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<td>First passage, day 3</td>
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<td>SA13/JFH1</td>
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<tr>
<td>SA13/JFH1C3405G-A3696G</td>
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<tr>
<td>SA13/JFH1G2611T-A2728G-C3405G-A3696G</td>
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**NOTE.** Nucleotide positions refer to SA13/JFH1. Uppercase and lowercase letters indicate dominant and nondominant nucleotides, respectively; double uppercase letters indicate that a dominant sequence was not determinable. Mutations representing at least a 50%/50% distribution in first passage are shown.

* Nucleotide positions are those of the H77 reference strain (GenBank accession number AF009606).

b The transfection is shown in figure 1A. In first-passage SA13/JFH1 viruses in another transfection experiment (shown in figure 1B), we found C3623T (Arg1095Trp) and C4972T (noncoding) present in a 50%/50% distribution.

c In 1 of 2 independent second passages, clonal analysis was performed. Only nucleotide changes found at the same position in at least 2 clones are shown. Additionally, in all analyzed clones, mutations occurring in only 1 clone were found.

d Used in neutralization assay (figure 4A).

e C4801A is noncoding; G4801T is coding (Gln1487His).

f In another transfection experiment, SA13/JFH1C3405G acquired T3954C (Val1205Ala) in a 50%/50% distribution.

g Mutation introduced into SA13/JFH1.

h Used in virus-treatment experiments, neutralization assays (figure 4A and 4C), and anti-CD81 and anti–SR-BI experiments (figure 3A and 3B).
served improved growth. To systematically compare the growth kinetics of passaged viruses, we infected Huh7.5 cells with equivalent doses of SA13/JFH1 and J6/JFH first-passage viruses (figure 1C); in contrast to what we had seen in the transfection experiment, we observed comparable infection spread, infectivity titers (peaking at $10^5$ TCID$_{50}$/mL), and RNA titers (peaking at $10^7.5$ IU/mL). As found for other JFH1-based recombinants [7, 8, 19], complete infection with the 5a/JFH1 virus was followed by massive cell death and a decrease in the percentage of HCV Core–positive cells (data not shown).

We reasoned that the improved growth kinetics of SA13/JFH1 might be caused by the acquisition of adaptive mutations. Thus, we determined the SA13/JFH1 open-reading-frame consensus sequence of viruses recovered from supernatants (table 1). In the first- and second-passage viruses, we found evidence of a total of 4 nucleotide changes in p7, NS2, and NS3 (G2611T, A2728G, C3405G and A3696G), resulting in amino acid changes in NS2 (A1022G) and NS3 (K1119R). Direct sequencing found that, at all positions, the original sequence was still present. To investigate how these mutations were combined on individual genomes, we performed clonal analysis of a 7-kb fragment of second-passage viruses; 5 of 10 clones were found to have all 4 of the nucleotide changes, whereas 4 clones were found to have the original sequence (table 1).

**Characterization of adaptive mutations leading to efficient growth of 5a/2a viruses.** To evaluate the effect of the identified SA13/JFH1 mutations, we performed transfection and first-passage experiments with SA13/JFH1C3405G, SA13/JFH1C3405G-A3696G and SA13/JFH1G2611T-A2728G-C3405G-A3696G; for comparison, we included
the original SA13/JFH1 and J6/JFH (figure 2A and 2B). During transfection, SA13/JFH1 required an adaptation phase of at least 13 days before achieving titers of $\sim 10^5$ TCID$_{50}$/mL (figure 2A; data not shown) and, in first-passage viruses, acquired an NS3 mutation (table 1), resulting in amino acid change I1313V. In contrast, all 3 mutated recombinants showed growth characteristics comparable to those of J6/JFH, with peak infectivity titers of $\sim 10^5$ TCID$_{50}$/mL in transfection and first-passage experiments (figure 2A and 2B) and with peak HCV RNA titers of $\sim 10^7.5$ IU/mL in first-passage experiments (figure 2B); SA13/JFH1$_{C3405G}$ and SA13/JFH1$_{C405G-A3696G}$ were genetically stable, whereas SA13/JFH1$_{G2611T-A2728G-C3405G-A3696G}$ acquired a synonymous mutation in NS5A (table 1).

**Inhibition of entry of genotype 5a viruses by blocking of CD81 and SR-BI.** In vitro infection of Huh7-derived cells by HCV genotypes 1–4 has been shown to depend on the cell-surface receptor CD81 [2–8], and entry of genotypes 1 and 2 has been shown to depend on SR-BI [20–22]. We demonstrated that entry of genotype 5a also depended on CD81, because preincubation of Huh7.5 cells with anti-CD81 antibodies resulted in a dose-dependent inhibition of infection with SA13/JFH1$_{C3405G-A3696G}$ virus, with $>90\%$ inhibition at 2.5 $\mu$g/mL (figure 3A). A dose-dependent inhibition of entry was also seen when Huh7.5 cells were preincubated with anti–SR-BI antibodies, with 97% inhibition at a 1:10 dilution (figure 3B).
For both receptor-blocking experiments, we tested inhibition of entry of J6/JFH1 in parallel and found comparable dose-dependent results (figure 3A and 3B).

**Efficient neutralization of genotype 5a viruses by chronic-phase sera and purified IgG from genotype 5a–infected patients.** We investigated whether sera of genotype 5a–infected patients could neutralize ~100 TCID \(_{50}\)'s of the homologous SA13/JFH1 virus (figure 4A and table 2). Autologous SA13 chronic-phase serum [9, 18], the source of the SA13 strain, exhibited >50% neutralization at a dilution of 1:1600. Two additional genotype 5a sera, SA1 and SA3 [18], showed >50% neutralization at dilutions of 1:25,600 and 1:6400, respectively. Thus, sera of patients chronically infected with genotype 5a had relatively high titers of neutralizing antibodies against a homologous 5a/JFH1 virus.

To rule out nonspecific inhibition by serum factors, we performed neutralization with purified IgG from the SA3 serum, which was available in sufficient quantity, and found a dose-dependent neutralization yielding 97% inhibition at 40 \(\mu\)g of SA3 IgG and >50% neutralization at 1.25 \(\mu\)g of IgG (figure 4B). We also investigated neutralization of IgG purified in parallel from chronic-phase serum of genotype 1a–infected patient H (H06), and we found that SA13/JFH1 was neutralized by a 50% neutralization titer of 1:25,600 [8], a potency mirrored by 98% inhibition at 40 \(\mu\)g of IgG and >50% inhibition at 0.625 \(\mu\)g of IgG (the lowest amount tested) (figure 4B).

**Cross-genotype neutralization potential of genotype 5a sera.** We recently have found that chronic-phase sera from genotype 1a– and genotype 4a–infected patients could cross-neutralize intergenotypic recombinant viruses of genotypes 1a, 4a, 5a, and 6a but did not neutralize recombinants of genotypes 2a and 3a [8]. In the present study, we examined the ability of 5a sera (2-fold dilutions starting at 1:100) to cross-neutralize intergenotypic recombinants of the different genotypes [3, 7, 8] (I.M.G., unpublished data). The SA1 serum had the highest reciprocal 50%–cross-neutralization titers, which were 1600, 400, and >51,200 against viruses of genotypes 1a, 4a, and 6a, respectively (table 2). The SA3 and SA13 sera had limited or no cross-neutralization activity against viruses of genotypes 1a and 4a, but both sera had relatively high titers of neutralizing antibodies against the genotype 6a virus (table 2). At the 1:100 dilution, the genotype 5a sera had no detectable cross-neutralization activity against viruses of genotypes 2a and 3a; however, when we subsequently tested a 1:50 dilution of the SA1 serum, which had the highest homologous neutralization titer, we observed >50% neutralization of viruses of genotypes 2a and 3a (data not shown).

**Treatment with homologous neutralizing antibodies: failure to prevent spread of SA13/JFH1 in vitro.** To investigate the treatment potential of neutralizing antibodies in vitro, we used SA3 serum, because of its availability and relatively high 50% neutralizing titer (1:6400) against SA13/JFH1. As shown in figure 4C, a 1:50 dilution of SA3 serum had a strong neutralizing effect, which, however, decreased with increasing doses of SA13/JFH1 C3405G-A3696G virus; thus, ~50, ~100, ~200, ~400, and ~800 TCID \(_{50}\)'s were neutralized with 97%, 97%, 86%, 90%, and 88% efficacy, respectively. We used ~100 and ~50 TCID \(_{50}\) SA3/JFH1 C3405G-A3696G preincubated with SA3 serum at dilutions of 1:200 (figure 5A) and 1:50 (figure 5B), respectively, to infect Huh7.5 cells. Subsequently, cells were treated every 24 h with SA3 serum containing medium; the reciprocal 50%–neutralization titers were ~32 (1:200 dilution) and ~128 (1:50 dilution), respectively.

Even though there was a delay in infection kinetics in both SA3–treated cultures compared with what we observed in the serum control (and virus-only control), these cultures also became fully infected after 10 days (figure 5A and 5B). In the culture treated with the lowest neutralizing-antibody dose, the consensus sequence of the envelope genes of released viruses at day 11 showed evidence of quasispecies at one position in HVR1 of E2 (C1500T/C), which encoded amino acid change T386I; in clonal analysis, this mutation was seen in only 1 of 3 clones. Furthermore, we could not detect any changes in the consensus sequence of genomes recovered at day 10 during treatment with

<table>
<thead>
<tr>
<th>Serum strain</th>
<th>Genotype 1a</th>
<th>Genotype 2a</th>
<th>Genotype 3a</th>
<th>Genotype 4a</th>
<th>Genotype 5a</th>
<th>Genotype 6a</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA1</td>
<td>1600</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>400</td>
<td>25,600</td>
<td>&gt;51,200</td>
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<tr>
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<td>&lt;100</td>
<td>&lt;100</td>
<td>200</td>
<td>6400</td>
<td>12,800</td>
</tr>
<tr>
<td>SA13</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>1600</td>
<td>3200</td>
</tr>
</tbody>
</table>

**NOTE.** Data are reciprocal 50%-neutralization titers. Neutralization of HCV was performed with ~100–200 TCID \(_{50}\)'s of JFH1-based recombinants containing Core–NS2 of genotypes 1–6. The recombinant viruses were incubated in triplicate with 2-fold serial dilutions of either chronic-phase sera of HCV genotype 5a–infected patients (SA1, SA3, and SA13) or a serum control, before being tested on Huh7.5 cells. Reciprocal neutralization titers were determined as the highest serum dilution showing a 50% reduction in focus-forming units (FFUs), compared with the average FFUs for a serum control. The average number of FFUs in the serum control in the different genotype experiments was 10–70 FFUs/well. In serum-control experiments for a particular genotype, there was no significant FFU difference between the different dilutions (data not shown), as also had been observed in a previous study using the same serum control [8].

[^75% neutralization at dilution 1:51,200.]: ~75% neutralization at dilution 1:51,200.
The 4-fold-higher neutralizing-antibody dose. During treatment with the higher dose (figure 5B), supernatant infectivity titers at days 2 and 4 were <10^1.7 TCID_{50}/mL. At day 7, the titer had increased to 10^0.7 TCID_{50}/mL, which still was lower than the titer of the control cultures. However, at day 9 we found similar infectivity titers for both the treated and the control cultures. Thus, the use of homologous neutralizing antibodies to treat HCV did not appear to prevent the latter’s spread in culture.

**DISCUSSION**

We have developed the first cell-culture system for HCV genotype 5, which is highly prevalent in South Africa and has been introduced into several regions in Europe [11–13]. In this 5a/JFH1 system, the 3' intergenotypic junction between NS2 and NS3 is located in the same region where it has been observed in naturally occurring recombinants [23, 24] and in JFH1-based cell cultures developed for genotypes 1–4 [3, 5–8].

As has been described for other intergenotypic recombinants [6–8], SA13/JFH1 adapted in culture. In 3 independent transfection experiments, the original SA13/JFH1 genome, when compared with J6/JFH, was significantly delayed in viral spread in Huh7.5 cells (figure 1A and 1B and figure 2A), and adaptive mutations acquired in culture accelerated infection kinetics (figure 1C and figure 2B). However, the original sequence at adapted positions was found in 4 of 10 clones derived from second-passage viruses of the original SA13/JFH1, suggesting that these particular mutations are not an absolute requirement for viability. In reverse-genetic experiments, we found that SA13/JFH1 viruses with a combination of amino acid changes in NS2 (A1022G) and NS3 (K1119R), mapping to the NS2–3 autoprotease domain [25], were genetically stable and yielded infectivity titers of ~10^5 TCID_{50}/mL, slightly higher than the titers observed for other JFH1-based intergenotypic recombinants [7, 8].

Amino acid changes in NS2 and NS3 are involved in cell-culture adaptation of other JFH1-based intergenotypic recombinants, possibly facilitating the interaction between genotypes. A genotype 4a recombinant depended on mutations in NS2 [8], whereas changes in NS3 conferred adaptation by genotype 1a [6, 8] and genotype 3a [7] recombinants. Interestingly, I1313V in type 4a recombinant depended on mutations in NS2 [8], possibly facilitating the interaction between genotypes. A genotype 5a recombinant depended on mutations in NS2 [8], whereas changes in NS3 conferred adaptation by genotype 1a [6, 8] and genotype 3a [7] recombinants. Interestingly, I1313V in type 4a recombinant depended on mutations in NS2 [8], possibly facilitating the interaction between genotypes. A genotype 5a recombinant depended on mutations in NS2 [8], whereas changes in NS3 conferred adaptation by genotype 1a [6, 8] and genotype 3a [7] recombinants. Interestingly, I1313V in type 4a recombinant depended on mutations in NS2 [8], possibly facilitating the interaction between genotypes.
the range expected to control HCV.

Additionally, we studied the neutralization potential of sera from genotype 5a–infected patients. As previously has been observed for genotype 1 sera and genotype 4 sera [8], genotype 5a sera efficiently neutralize the homologous genotype 5a virus, as well as genotype 6a virus, but not genotype 2 and genotype 3 viruses. Neutralization efficiency against genotype 1 and genotype 4 viruses was intermediate and varied between the genotype 5a sera. The fact that purified IgG from genotype 1a sera and genotype 5a sera could efficiently neutralize SA13/JFH1 shows that the neutralization obtained with serum was not due to nonspecific inhibition of entry of HCV by serum-derived molecules. The results of the present study suggest a closer serological relationship between genotypes 1, 4, 5, and 6, as also has been seen in the pseudoparticle system [16], but they also suggest a genotype- and strain-specific difference in the magnitude of this neutralization, indicating that it will be a challenge to develop vaccines that induce high titers of broadly reactive neutralizing antibodies.

Even though neutralizing antibodies might not clear HCV infection in vivo [16, 28–30], highly potent neutralizing antibodies are expected to be of importance for the development of anti-HCV immunotherapies, applicable as postexposure prophylaxis after needle-stick injuries and as reinfection prophylaxis after liver transplantation in HCV-infected patients, as well as for the development of an HCV vaccine [31, 32]. In the present study, we have shown in vitro that even low-dose viral infections could not be controlled by continuously treating SA13/JFH1 cultures with homologous neutralizing antibodies, which yielded efficient neutralization in the in vitro neutralization test. Because we were not able to achieve 100% neutralization in the neutralization assays (figure 4C), either a slow spread due to nonneutralized virus or cell-to-cell spread might have occurred during the initial phase of treatment [33]. During the course of treatment, viral titers might have out-competed the applied dose of antibodies, because relatively high infectivity titers were measured in the supernatant after a few days of treatment (figure 5A and 5B). We have shown that neutralization efficiency depends on the viral dose (figure 4C), and, in vivo, rather high doses of neutralizing antibodies seem to be necessary for protection [34]. However, in vivo, passive immunization with anti-HCV IgG has been found to protect uPA-SCID (urokinase plasminogen activator–severe combined immunodeficient) mice engrafted with human hepatocytes against HCV infection, and sera from the animals at the time of challenge have been found to have reciprocal 50%-neutralization titers of 100–400 in vitro [35]. Although some of these mice had breakthrough infections, with slower viral kinetics, these data indicate that the dose of neutralizing antibodies used in the treatment experiments should be in the range expected to control HCV.

Even though viral escape has been described to occur in vivo [30], it is unlikely to be responsible for the treatment failure that we observed, because we could not detect amino acid changes in the envelope proteins of viruses recovered after the virus had spread through the entire culture. In line with the results of the present study, high-titer neutralizing antibodies against the homologous virus have not always been found to be able to prevent HCV infection in uPA-SCID mice engrafted with human liver [34, 35], and these infections have been shown to be due to neutralization failure and not to viral escape [35]. Furthermore, in the presence of neutralizing antibodies in vitro, JFH1 viruses have been reported to spread without employing obvious genetic-escape mechanisms, because intracellular virus recovered after spread under neutralization pressure could still be neutralized by the same neutralizing antibodies that had been used for treatment [33]. Identification of sera with higher neutralization titers, high titer IgG preparations, or efficient monoclonal antibodies could perhaps permit more efficient treatment in vitro and in vivo.

In summary, we created a cell-culture system for genotype 5a, with Core–NS2 of strain SA13 in the backbone of JFH1. We identified mutations in NS2 and NS3 that led to the highest infectivity titers among JFH1-based intergenotypic recombinants reported thus far. The present study confirms the possibility that cross-genotype neutralization may provide a potential advantage in prophylactic and treatment strategies. Unfortunately, the present study also found that treatment of HCV cultures during the initial stages of a low-dose infection with relatively high-titer (reciprocal 50%-neutralization titer, >100) homologous neutralizing serum concentrations could not sustain suppression of SA13/JFH1 infection in vitro, underlining the complexity and limitations of the use of neutralizing antibodies in therapeutic strategies to treat HCV.

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