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Immunoglobulin with High-Titer *In Vitro* Cross-Neutralizing Hepatitis C Virus Antibodies Passively Protects Chimpanzees from Homologous, but Not Heterologous, Challenge

Jens Bukh,[a,b] Ronald E. Engle,[b] Kristina Faulk,[b] Richard Y. Wang,[c] Patrizia Farci,[b] Harvey J. Alter,[c] Robert H. Purcell[b]

Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases and Clinical Research Centre, Hvidovre Hospital, and Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark[a]; Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA[b]; Department of Transfusion Medicine, Grant Magnuson Clinical Center, National Institutes of Health, Bethesda, Maryland, USA[c]

The importance of neutralizing antibodies (NAbs) in protection against hepatitis C virus (HCV) remains controversial. We infused a chimpanzee with H06 immunoglobulin from a genotype 1a HCV-infected patient and challenged with genotype strains efficiently neutralized by H06 *in vitro*. Genotype 1a NAbs afforded no protection against genotype 4a or 5a. Protection against homologous 1a lasted 18 weeks, but infection emerged when NAb titers waned. However, 6a infection was prevented. The differential *in vivo* neutralization patterns have implications for HCV vaccine development.

Chimpanzees have been essential for hepatitis C virus (HCV) research (1–3). Understanding the role of neutralizing antibodies (NAbs) in preventing HCV infection is important for vaccine efforts against this important pathogen, which annually infects ~4 million people and causes chronic liver disease (4–12). We showed that chronic-phase patient serum/plasma prevented HCV infection of chimpanzees if the virus and anti-HCV antibody were incubated prior to inoculation (13, 14). Others showed that immunoglobulin (IgG) purified from anti-HIV antibody-positive blood donors prevented HCV infection when mixed with virus and then administered to a chimpanzee (15). However, the protective effect of preexisting polyclonal HCV NAbs remains to be determined.

Studies with culture systems using pseudotyped particles (HCVpp) or infectious viruses (chimeric cell culture-derived HCV [HCVcc]) confirmed the existence of NAbs in acute- and chronic-phase patients, with high-level cross-neutralization of heterotypic HCV strains (16–20). However, differences exist in neutralization capacities of chronic-phase samples against HCV variants of the same genotype (21), and there is a constant evolution of HCV variants escaping NAbs (14, 22, 23). Nonetheless, these *in vitro* data suggest that chronic-phase HCV samples might be useful for broad protection against HCV. Effective NAbs could help define critical epitopes for vaccine development (24–27).

We prepared H06 IgG from plasma obtained almost 30 years after disease onset in patient H with persistent HCV infection (28). By infusing H06 IgG with high *in vitro* neutralizing titers into a chimpanzee (CHA5A009) and then challenging with different HCV strains sensitive to neutralization *in vitro* (18, 19), our aim was to test the principle of passive immunoprophylaxis against homologous and heterologous strains *in vivo*.

Animal experimentation and sample collection from chimpanzee CHA5A009 were performed from 2007 to 2008. The housing and care of the chimpanzee met or exceeded all requirements of the National Research Council's 1996 *Guide for the Care and Use of Laboratory Animals*, 7th ed. (National Academies Press, Washington, DC), which were in effect until 2010. The project license (ASP LID 64) and specific protocol (06-C-482) were separately approved by the Institutional Animal Care and Use Committees of the National Institute of Allergy and Infectious Diseases, NIH, and Bioqual, Inc., the facility housing the chimpanzee. Both facilities were fully accredited by the American Association for Accreditation of Laboratory Animal Care International. Specifically, housing exceeded requirements for square footage and cage height for the chimpanzee's size. Feeding consisted of approved chimpanzee biscuits with supplemental vegetables and fruit and *ad libitum* water. A comprehensive enrichment program was in place and consisted of foods, puzzles, and environmental enrichment, coupled with visual and auditory contact with other chimpanzees. Sample collection was performed under ketamine hydrochloride anesthesia. Euthanasia was not required.

CHA5A009 was infused with H06 IgG intravenously prechallenge (250 mg/kg body weight) and at weeks 2.5 and 3.5 postchallenge (125 mg/kg). Twenty-four hours after the first IgG infusion, CHA5A009 was challenged intravenously with 100 chimpanzee infectious doses of each of the HCV strains (with the genotype in parentheses) H77(1a), ED43(4a), SA13(5a), and HK6a(6a) (29–31), chosen because H77 is the homologous acute-phase patient H strain (31) and chronic-phase patient H sera cross-neutralized all strains in HCVpp and HCVcc assays (18, 19). Based on 5′-untranslated region (UTR)-based TaqMan assay (32), CHA5A009 had serum HCV titers of 2.7 and 4.8 $\log_{10}$ IU/ml at weeks 1 and 2, respectively, and peak titers of 5 to 6 $\log_{10}$ IU/ml at weeks 3 to 11; the animal remained persistently infected (Fig. 1A). 5′-UTR (33) and core-envelope 1 (core-E1) (34) analysis of week 2 viruses,

Received 9 May 2015 **Accepted** 15 June 2015

Accepted manuscript posted online 17 June 2015

Citation Bukh J, Engle RE, Faulk K, Wang RY, Farci P, Alter HJ, Purcell RH. 2015. Immunoglobulin with high-titer *in vitro* cross-neutralizing hepatitis C virus antibodies passively protects chimpanzees from homologous, but not heterologous, challenge. J Virol 89:9128–9132. doi:10.1128/JVI.01194-15.

Editor: M. S. Diamond

Address correspondence to Jens Bukh, jbukh@sund.ku.dk.

doi:10.1128/JVI.01194-15

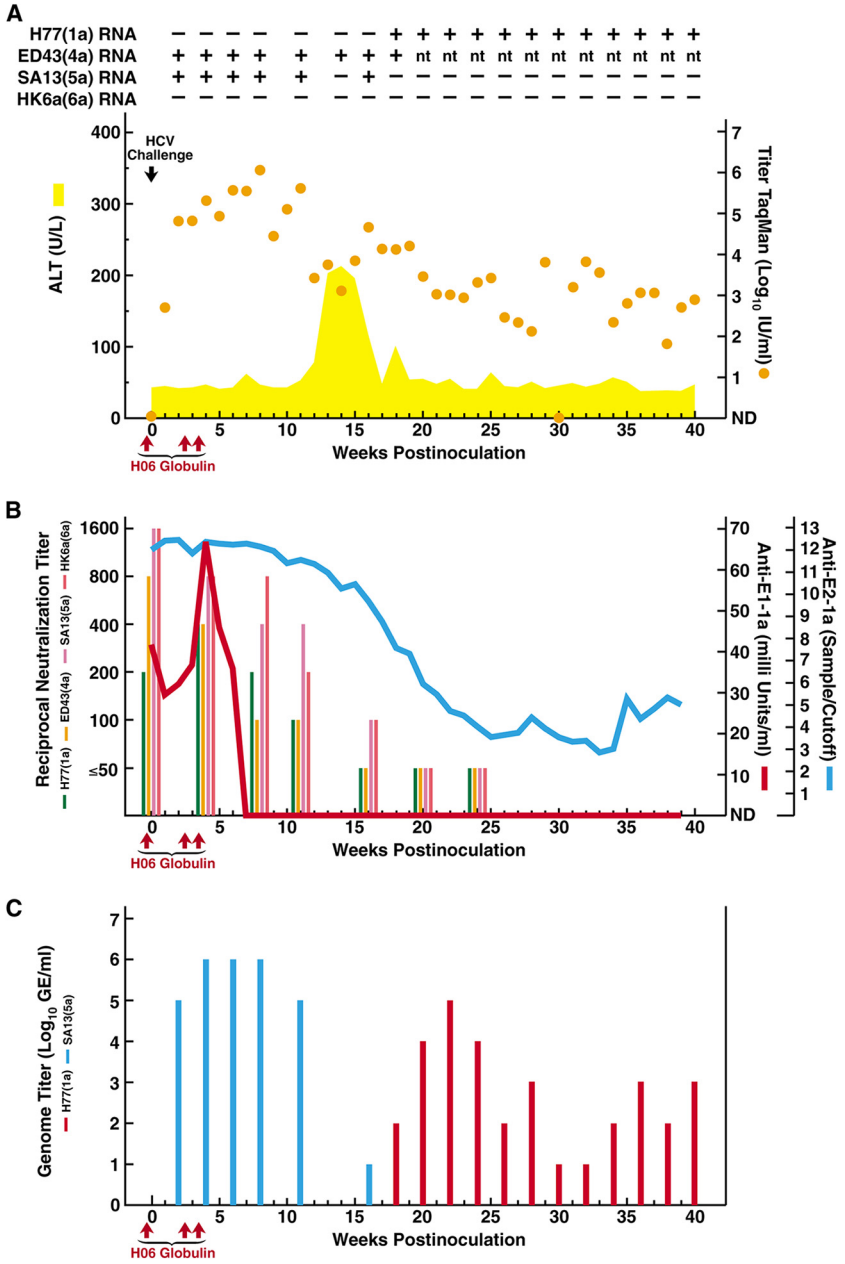

FIG 1 Course of hepatitis C infection in a chimpanzee loaded with polyclonal genotype 1a immunoglobulins and challenged with homologous and heterologous HCV strains. Chimpanzee CHA5A009, who was naive for HCV, as well as for other hepatitis viruses, was infused intravenously with immunoglobulins (H06), prepared from plasma obtained from patient H with chronic HCV, at the time points indicated with red arrows; the first infusion was 24 h before HCV challenge. The intravenous challenge (indicated by a black arrow) was 100 chimpanzee infectious doses of each of the HCV strains (with genotype in parentheses) H77(1a), ED43(4a), SA13(5a), and HK6a(6a). (A) Serum samples were tested for HCV RNA by in-house RT-nested PCR with strain-specific primers (Table 1): +, positive; −, negative; nt, not tested. The estimated $\log_{10}$ HCV RNA titers (international units per milliliter), determined in an in-house TaqMan assay (orange dots), were plotted against time; a single sample below the detection limit of ~10 IU/ml is shown as "not detected" (ND). The area shaded yellow shows serum ALT (units per liter). Results of samples collected after week 40, at weeks 50 and 56, are only referred to in the text. (B) Anti-HCV antibodies against E1 (red line) and E2 (blue line) were detected in serum by 1a-specific ELISAs. The serum neutralizing reciprocal antibody titers, determined in HCVpp assays using H77, ED43, SA13, and HK6a pseudoparticles, are shown in colored bars as indicated. (C) The serum H77(1a) and SA13(5a) genome titers, determined by in-house RT-nested PCR with strain-specific primers (Table 1) on 10-fold serially diluted RNA, are indicated by red and blue bars, respectively.

amplified by reverse transcription (RT)-nested PCR detecting the four strains with equal sensitivity (29, 33, 34), detected only SA13(5a). The animal developed acute hepatitis with elevated serum alanine aminotransferase (ALT) levels from weeks 12 to 18 (Fig. 1A).

Serum anti-E2 by enzyme-linked immunosorbent assay (ELISA) (18) was saturated prechallenge through week 16 post-challenge, followed by a steady decrease in titers (Fig. 1B). Anti-E1 (INNO-LIA-HCV) (35) was detected prechallenge through week 6 (Fig. 1B). Serum NAbs were assayed in HCVpp assays using H77, ED43, SA13, and HK6a pseudoparticles, respectively (18). Serum

TABLE 1 Primers used in the HCV strain-specific RT-nested PCR in this study

Strain[a] and primer type	Primer	Sequence
H77(1a)		
RT	2832R-H77	AAGCGCCCCTAACTTGATGATG
PCR I	2427S-H77	ACTGGACACGGAGGTGGCCGCGTC
	2832R-H77	AAGCGCCCCTAACTTGATGATG
PCR II	2462S-H77	TTGTTCTTGTCGGGTTAATGGCGC
	2645R-H77	GGGTGTACTACACACATGAGTAAG
ED43(4a)		
RT	HCV4aCoseR3591	CCGTGGTAGACGGTCCACATCAC
PCR I	2676F-ED43	AGGGCCGGTTCCCAGCTGCT
	HCV4aCoseR3591	CCGTGGTAGACGGTCCACATCAC
PCR II	2839F-ED43	GCACTACAAGTTATGGCTGGCTA
	HCV4aCoseR3318	CACGCAGCGGTGTCAGCGCCCC
SA13(5a)		
RT	917R-SA13	CAGTTGCAGTCCTGCACAACATTA
PCR I	741S-SA13	AGCCCCGAGCCTCGGAGCGGT
	917R-SA13	CAGTTGCAGTCCTGCACAACATTA
PCR II	748S-SA13	AGCCTCGGAGCGGTCACGGCT
	898R-SA13	CATTATGCCGGCGAGGGCTAT
HK6a(6a)		
RT	1765R-HK6a	TGGGGCACAAAAGCTCATTGG
PCR I	1405S-HK6a	TGGGGCCAAATAACCTACAAA
	1765R-HK6a	TGGGGCACAAAAGCTCATTGG
PCR II	1408S-HK6a	GGCCAAATAACCTACAAAGTC
	1762R-HK6a	GGCACAAAAGCTCATTGGCAG

[a] Genotypes are given in parentheses.

taken prior to IgG infusion had reciprocal neutralization titers of <50 against all HCVpp strains. Following IgG infusion, CHA5A009 had significant NAb titers against all strains, remaining at ≥100 through week 11; at weeks 20 and 24, the titers were ≤50 for all strains (Fig. 1B).

Sera from weeks 2 to 56 were tested for H77, ED43, SA13, and HK6a HCV RNA by RT-nested PCR (Fig. 1A), using strain-specific primers (Table 1) (36). The sensitivities of the H77, SA13, and HK6a assays were equivalent to that using 5′-UTR primers when testing dilutions of H77, SA13, and HK6a pools, respectively (30); the 4a assay was suboptimal. Amplicons were in most cases sequenced, confirming strain authenticity. There was no evidence of H77(1a) infection until week 18, when H77 RNA was detected at 2 $\log_{10}$ genome equivalents (GE)/ml (Fig. 1A and C) (36). The H77(1a) emergence followed the decrease in H77 NAb titers to <50 (Fig. 1B). The week 18 H77(1a) envelope proteins, derived from overlapping amplicons (37), had no changes compared to the polyclonal challenge virus (38, 39), indicating that viral emergence was not due to escape mutations. The animal remained H77 infected, with titers of 4 to 5 $\log_{10}$ GE/ml from weeks 20 to 24; titers fluctuated at low levels thereafter (Fig. 1C).

Despite relatively high NAb titers against SA13(5a) (Fig. 1B), there was no apparent *in vivo* protection against this strain, which appeared at 5 to 6 $\log_{10}$ GE/ml in samples from weeks 2 to 11 (Fig. 1C); infection resolved after week 16 (Fig. 1A and C). The envelope sequence of week 2 SA13(5a), derived from overlapping amplicons (29, 37), had no changes compared to the polyclonal challenge virus (29, 40), indicating failure of neutralization. CHA5A009 became ED43(4a) infected, with sequence-confirmed ED43 RNA positivity from weeks 2 to 18 (Fig. 1A); since we did not develop a 4a-specific assay with optimized sensitivity, titer or outcome analysis was not performed. Finally, the chimpanzee apparently was not infected with HK6a(6a), being HK6a RNA negative from weeks 2 to 56 (Fig. 1A).

This is the first study to address whether polyclonal NAbs, given prechallenge, can prevent HCV infection in chimpanzees. While the homologous virus was suppressed initially, there was failure of neutralization against 2 of 3 heterologous HCV strains. Furthermore, the homologous virus emerged following the disappearance of passively administered NAbs and led to persistent infection. This supports the observation that polyclonal antibodies given postchallenge could control HCV infection only temporarily in the chimpanzee model (41). The infused NAbs might have prevented infection with heterologous 6a virus, and this was the strain previously found to be most efficiently neutralized in HCVcc assays (19, 42).

We previously demonstrated that chronic-phase patient H IgG, infused prechallenge, could control H77 infection in human liver chimeric mice (28, 43). However, the animals could only be followed short term. The reciprocal *in vitro* neutralization titers detected prechallenge and at weeks 4 and 8 in CHA5A009 (Fig. 1B) were similar to those detected prechallenge in human liver chimeric mice (43). Thus, for the homologous strain, it appeared that polyclonal immunoglobulin with *in vitro* neutralizing activity can control viremia *in vivo*. The chronic-phase sera from patient H had high *in vitro* neutralization titers against the heterologous 4a, 5a, and 6a strains (18, 19), and we previously found partial protection in human liver chimeric mice loaded with H06 IgG against ED43(4a) and HK6a(6a) (28). Here we observed reciprocal neutralization titers of >400 prechallenge and at week 4 postchallenge in the H06-infused chimpanzee using the same HCVpp assays for these 3 strains. Yet, the chimpanzee became infected with the 4a and 5a strains and developed acute hepatitis. For the 5a strain, we showed that this could represent failure of neutralization, since the challenge virus without envelope mutations appeared at high titers at week 2. In evaluating the outcome for the 1a, 4a, 5a, and 6a challenges, it should be recognized that the HCVpp neutralization titers in the chimpanzee were lower than those previously detected for the different genotype stains in chronic-phase sera from patient H (18). Furthermore, it must be recognized that our study in a single chimpanzee does not necessarily permit broad conclusions.

In a recent study, prechallenge infusion with 250 mg/kg of a humanized monoclonal anti-E2 prevented infection in an H77-challenged chimpanzee (44). Using polyclonal immunoglobulin with an HCV-specific antibody concentration of <250 mg/kg, we observed control of H77 viremia but not sterilizing immunity, even though we also infused the animal with IgG postchallenge. In our study, viral interference could have influenced the outcome given the simultaneous infections with 4a and 5a strains.

Recent data indicate that the HCV clearance is associated with NAbs (23, 45, 46). During chronic infection, HCV persists despite the presence of high-titer NAbs, perhaps because it is shielded from neutralization (47–50) or neutralization-resistant mutants are continuously developing (22, 23, 51). It was demonstrated *in vitro* that chronic-phase patient H sera neutralized only variants from time points early in infection, not later variants (22). Our data confirm *in vivo* that chronic-phase patient H immunoglob-

ulin can control the acute-phase virus. We previously showed that different recombinant genotype viruses, lacking HVR1, were efficiently neutralized by H06 IgG, indicating that it targets conserved neutralization epitopes within E1 and/or E2 (42).

This study sheds new light on the effectiveness of NAbs in preventing HCV. It is encouraging that the homologous virus apparently could be suppressed long term. However, the lack of protection against heterologous strains highlights significant issues regarding the possibility of developing broadly protective immunoglobulin preparations using patient-derived antibodies alone. More likely, efficient neutralization will require monoclonal antibodies against conserved conformational epitopes and/or combinations of antibodies targeting different HCV epitopes or viral variants (44, 50, 52–54).

ACKNOWLEDGMENTS

We thank Alicia Brockington for technical assistance and Charlene Shaver for animal care.

This study was supported by the Intramural Research Program of the NIAID, NIH, and by NIAID contract NO1-AO-62713, as well as by research grants to J.B. from the Danish Council for Independent Research-Medical Sciences, the Lundbeck Foundation, and the Novo Nordisk Foundation. J.B. is the recipient of an Advanced-Top Researcher grant from the Danish Council for Independent Research (2014) and the 2015 Novo Nordisk Prize.

The authors declare that they have no conflicts of interest to report.

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