Infectivity in chimpanzees (Pan troglodytes) of plasma collected before HCV RNA detectability by FDA-licensed assays: implications for transfusion safety and HCV infection outcomes

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Running Head: HCV Infectivity during acute infection

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Abstract

Serial plasma aliquots (50mL) obtained from 10 commercial donors who converted from HCV RNA negative to positive were transfused into two chimpanzees to assess infectivity during early HCV infection. Plasma, obtained 4 days prior to HCV RNA detectability by licensed assays, transmitted HCV infection to chimpanzee X355. The infectious PCR-negative plasma was subsequently shown to be positive in 2 of 23 replicates using a sensitive TMA assay, and estimated to contain 1.2 HCV RNA copies/mL (60 copies/50 mL transfused). Plasma units obtained up to 8 weeks earlier were not infectious in a second susceptible chimp, even when from donors with low-level, intermittent HCV RNA detection. Chimp x355 developed acute viremia with subsequent seroconversion, but cleared both virus and antibody in 17 weeks. When rechallenged 38 months later with 6,000 RNA copies/mL from the same donor, X355 was transiently reinfected and again rapidly lost all HCV markers. We conclude that: 1) transfusions can transmit HCV infection prior to RNA detection, but the interval of test-negative infectivity is very brief; 2) early “blips” of HCV RNA appear non-infectious and can be ignored when calculating residual transfusion risk; 3) markers of HCV infection can be lost rapidly after exposure to low-dose inocula.
Introduction

The phase between the onset of hepatitis C virus (HCV) infection and sustained systemic viremia is known as the eclipse or pre-viremic window phase of infection. At its conclusion, plasma HCV RNA concentrations rise exponentially in what has been termed the ramp-up phase of infection. The eclipse phase, by definition, is characterized by lack of detectable plasma viremia by commercially available HCV RNA assays, which are primarily based on polymerase chain reaction (PCR) technologies. However, by performing quadruplicate analyses of plasma donations from acutely infected source plasma donors using more sensitive qualitative transcription-mediated-amplification (TMA) assays for HCV RNA, we found previously that 108 of 225 eclipse phase donations unexpectedly demonstrated the presence of intermittent, low-level HCV RNA. A second study of source plasma donors found a similar phenomenon, as did studies in injection drug users (IDU) and transfusion recipients. Thus, HCV dynamics in the eclipse phase may follow one of two patterns: no viremia until a sustained rapid increase in serum RNA levels occurs (ramp-up) or intermittent low-level HCV RNA detection (previously referred to in HIV [human immunodeficiency virus] and SIV [simian immunodeficiency virus] infections as “blip” viremia) that precedes ramp-up by varying intervals up to two months.

Because HCV RNA levels are very low in this very early phase of infection, it has not been possible to determine if the RNA is contained in infectious virions or to characterize the sequence integrity of HCV RNA genomes during these “blips” relative to the sequences observed during the ramp-up period from the same subjects. With respect to blood safety, if the “blips” are found to be infectious, then current residual risk modeling might be underestimating HCV transfusion-transmission risk, since such modeling assumes that donations given prior to the extrapolated beginning of the ramp-up period are non-infectious.

The chimpanzee model of HCV infection has played a key role in our understanding of determinants of viral transmission and replication, the development of anti-viral immune responses, assessment of potential viral and host factors that lead to resolved versus chronic infection, and evaluation of therapeutics and vaccines. Of particular note, the chimpanzee model is considered an extremely sensitive model for assessment of parenteral infectivity relevant to
transfusion safety.\textsuperscript{13-24} For this reason, we conducted multi-step inoculation experiments in two chimpanzees, designed to study the infectivity of serially collected plasma donations from source plasma donors recently infected with HCV. First, we evaluated infectivity from donations initially assessed as occurring immediately prior to the onset of ramp-up phase viremia. Next we evaluated infectivity of non-viremic and low-level HCV RNA positive (blip) donations from donors who demonstrated intermittent HCV RNA detection in the eclipse phase of HCV infection. We also studied the early immunology of HCV infection following a low dose exposure and the influence of this exposure on subsequent reinfection with the same HCV isolate. In addition to contributing to our understanding of transfusion-transmission, these experiments provide data relevant to broader issues of HCV transmissibility, the ability to document previous HCV infection using RNA and antibody diagnostics, and the early immunological events in HCV infection.
Materials / Methods

Preparation of HCV-RNA Positive Plasma Donor Panels

Plasma for chimp inoculation studies was selected from a collection of 50 source plasma panels used in a previous study of early HCV viral dynamics. (see supplemental materials). Each panel consisted of aliquots of a series of donations (serial donations) from a given donor. These donations occurred at approximately twice weekly intervals in both the eclipse and ramp-up phases of acute HCV infection. We based the classification of ramp-up phase infection on the specific rate of increase in HCV viral load for each individual donor. We then back extrapolated from the slope of ramp-up viremia for each donor to establish a time when the estimated viral load would have been <0.05 copies/mL; all RNA-positive donations collected prior to this time point were classified as occurring in the eclipse phase; i.e., prior to the extrapolated beginning of the ramp-up phase. Donations given prior to quantifiable ramp-up viremia were then further evaluated for HCV RNA by performing TMA testing (Procleix discriminatory HCV [dHCV] assay, Gen-Probe, San Diego, CA) on four replicates of 0.5 mL of plasma for each donation. Multiple additional replicates (n = 20 or 23) of selected eclipse phase samples were also further tested for HCV RNA either by the dHCV assay or by the Procleix duplex (HIV-HCV) assay (Gen-Probe, San Diego, CA) as part of a previously published study. Based on this testing, we characterized 37 of the 50 panels as having intermittent HCV RNA positive donations (at least one dHCV TMA-positive replicate result) during the eclipse phase interspersed with non-viremic (all replicates negative by TMA) donations.

Selection of Plasma Donor Panels for Infusion into Chimps

We selected 10 ABO compatible serial donation panels for chimpanzee infectivity studies. We used five of the 13 panels that did not have evidence of intermittent viremia as well as five of the 37 that showed intermittent HCV RNA detection (blips). The five cases without intermittent viremia were selected to have the most frequent serial donations over the three weeks preceding RNA detection by quantitative HCV PCR and qualitative HCV TMA assays. The five cases with intermittent HCV RNA during the eclipse phase were selected to have at least three reactive
results among the four replicate HCV TMA results on two or more specimens collected more than two weeks prior to ramp-up viremia, as well as to include intervening donations that tested negative on a total of up to 27 replicate TMA assays.

**General Format of Chimp Inoculation Experiments**

Two healthy adult female chimpanzees (X331 and X355) were housed in a containment facility maintained by the Texas Biomedical Research Institute and Southwest National Primate Research Center, an AAALAC accredited facility. Housing conditions and animal manipulations were approved by the IACUC and all animal procedures were performed with prior administration of appropriate sedatives and anesthesia. Fifty mL of donor plasma units were intravenously administered; these could be either individual unit infusions, multiple units infused one after another, or a pre-mixed pool of 5 units (250 mL total volume). Further details of the timing of the infusions in the various experiments are provided in Figure 1, supplemental Figure 1, and in the results section.

The animals were monitored by testing blood samples for HCV RNA by PCR, anti-HCV by EIA, liver enzyme levels, and T cell assays. If infected, weekly or biweekly monitoring continued; however, if there was no detectable infection after 6 weeks, the animal was rested for an interval of up to 3 weeks and then became eligible for infusion with another donor plasma sample. This sequence was repeated with several different donor plasma samples until evidence of infection could be ascertained. Following confirmation of infection, animals were monitored for a period of one year to determine the outcome of infection. No additional infusions with donor plasma were performed until completion of this one-year period.
Laboratory assays

HCV RNA quantification in plasma donor panels

Two different assays were performed as previously described. The COBAS Amplicor HCV Monitor, Version 2.0 assay (Roche Molecular Systems, Pleasanton, CA) HCV PCR assay was able to quantify HCV RNA down to a lower limit of 600 IU/mL. Samples that were negative on this assay were tested using the dHCV TMA assay using multiple 0.5 mL replicates of plasma; limit of detection (LOD) of each replicate assay is 12.1 copies/mL (50% LOD; 95% confidence interval 11.1-13.2). HCV RNA concentration was determined based on the percentage of replicates that gave positive results using probit analyses.

HCV RNA detection and quantification in the recipient chimps

HCV RNA detection was performed using the COBAS Amplicor Hepatitis C Virus Test, version 2.0 (Roche Molecular Systems, Pleasanton, CA) and quantification of HCV RNA was performed using the COBAS Amplicor HCV Monitor, Version 2.0 assay.

HCV antibody detection

Initial screening of plasma donor panels was done using a 3rd generation HCV antibody EIA (Ortho Diagnostics, Raritan NJ). Repeat reactive samples were further evaluated by the Recombinant Immunoblot Assay version 3 (RIBA; Novartis Diagnostics, Emeryville CA). Initial screening of chimp sera was done using an anti-HCV 2.0 EIA (Abbott Laboratories, Abbot Park, IL) with confirmation by RIBA.

HCV RNA extraction, amplification, cloning, sequencing and phylogenetic analysis

HCV RNA was extracted from 140uL of plasma from one viremic time point from each of the five plasma donors implicated in HCV transmission to chimp X331 and from two viremic time points from the infected chimp using the QIAamp Viral RNA Mini Kit following the
manufacturer’s instructions (Qiagen Inc., Valencia, CA). Extraction and amplification of donor and chimp samples were performed on different days to eliminate the possibility of cross-contamination. Details of the amplification, cloning, sequencing, and phylogenetic analysis procedures are in the supplemental materials.

**ALT and AST measurements**

Serum samples collected from the study animals were analyzed for AST and ALT levels with a Unicel DXC600 serum chemistry analyzer (Beckman Coulter). Enzyme levels were considered normal if they were within the normal range (AST 11–25 U/L; ALT 21–55 U/L) established at the primate center.

**CD4 proliferative responses to HCV antigens:**
Peripheral blood mononuclear cells (PBMC) were isolated from ACD-anticoagulated chimpanzee blood via gradient centrifugation as described. Triplicate cultures of 200,000 PBMC were stimulated with 1 µg/mL HCVcore, NS3, helicase, NS4, NS5A, NS5B proteins (Mikrogen, Germany) or buffer control as previously described. Cultures were labeled with 1 µCi [³H]thymidine (Amersham) on day 5 and harvested 16h later. Separate cultures stimulated with or without PHA (1 µg/mL, Murex Biotech Limited) were labeled with [³H]thymidine on day 2. The stimulation index (SI) was calculated as a ratio of the average number of counts per minute of four replicate cultures in the presence of antigen as compared to control buffer or medium.
Results

Three chimp inoculation experiments were performed in multiple phases as described below and in Figure 1.

**Experiment I: Testing the Infectivity of Plasma Immediately Prior to the Ramp-up Phase**

Experiment I, Phase I involved infusions of 50 mL of plasma (during a single infusion episode) from each of 5 different source plasma donors into an HCV naïve chimp (X355). The infused donations were from donors in whom there was no HCV RNA detected during the eclipse phase and were selected so as to be the donations that immediately preceded the first ramp-up viremia donation. (Figure 2 panel A, and supplementary Figure 1 panels A-E). These donations, which tested negative for HCV RNA by quadruplicate dHCV TMA and viral load assays, were estimated by back-extrapolation from measured viral loads during the subsequent ramp-up phase of each donor to have been collected from four to 16 days prior to the onset of the ramp-up phase (defined as one HCV RNA copy present in 20 mL of plasma).

After infusion, chimp X355 was evaluated weekly for the first 11 weeks, then biweekly until week 29 post-infusion, with a final assessment at one year post-infusion. Tests performed were HCV RNA (qualitative and, when indicated, quantitative), HCV antibody, ALT, and AST. Assays for PBMC proliferative responses to HCV proteins were performed prior to infusion and biweekly until week 10 post-infusion. As shown in Figure 3, chimp X355 developed mild hepatitis (peak ALT 68 IU/L) and transient HCV infection with viremia developing at week 2 and persisting until week 9. Peak viral load was $5.2 \times 10^4$ copies/mL at week 4. HCV antibody was first detected at week 8 and persisted until week 17, after which it became undetectable throughout follow-up (seroreversion). Samples collected from weeks 9 through 13 were RIBA positive but never showed antibody reactivity to the core (c22) antigen. Samples at weeks 15 and 17 showed very low antibody reactivity by RIBA (c33 at 1+, 511 and NS5 at +/-, and c22 non-detectable). ALT was elevated from weeks 5 through 8 with a peak value of 68 U/L. Proliferation of PBMC to HCV antigens was not detectable pre-infusion or at week 2, but a clear peak was detectable at week 4. Responses were exclusively targeted against nonstructural HCV.
antigens as evidenced by stimulation indices of 33 and 27 against NS5B and NS5A respectively, 29 against NS4 and 31 against NS3. Responses were maintained at week 6 and 8 post infusion, and declined to slightly above baseline at week 10. By 17 weeks post-infusion, HCV RNA and HCV antibody were undetectable and ALT levels were normal, thus leaving no residual evidence of prior HCV infection except for the cell-mediated immune response which remained detectable as late as week 21 post infusion.

Sequencing of HCV RNA and phylogenetic analysis revealed that the chimp HCV isolate was very closely related to an HCV isolate obtained from a subsequent highly viremic donation from one of the five source plasma donors used in the infecting inocula (Figure 4). The transmitting donation had been collected four days prior to the first quantifiable HCV RNA positive donation (viral load of $6 \times 10^3$ HCV RNA copies/mL) from this donor (donor 10081). In order to increase the sensitivity for detection of HCV RNA in the transmitting and non-transmitting source plasma donations, 23 additional 0.5 mL plasma replicates from the infused donations were tested under code for HCV RNA by the TMA assay. In total, two of the 27 replicate TMA assays were positive with plasma from the donation implicated as infectious by phylogenetic analysis, whereas similar multiple replicate TMA testing performed on identically processed frozen-thawed plasma from the other four donations in the transmitting pool gave negative results (Table 1). A probit analysis indicated that the dHCV TMA positive donation had an estimated HCV RNA concentration of 1.2 copies/mL; 95% CI: 0.5 – 1.9 copies/mL. In retrospect, this initially HCV RNA negative donation was probably in the very early ramp-up phase of infection such that multiple replicate testing was required to detect the very low-level of HCV RNA that was present. Since 50 mL of plasma from this donation was infused, we estimate that chimp X355 acquired HCV infection after being transfused with a total of approximately 60 HCV RNA copies; 95% CI: 25 – 95 copies.

To confirm that the donation that initially infected chimp X355 was the earliest infectious donation in the panel of serial donation samples from the implicated donor, we conducted Phase II of this experiment. A second HCV naïve chimp (chimp X331) was infused on separate occasions with 50 mL plasma from HCV RNA negative units donated by the transmitting donor that had been collected 14, 10, 7, and 3 days prior to the transmitting donation (Figure 2, panel
B). Chimp X331 was monitored weekly for 6 weeks following each 50 mL infusion (total of 24 weeks) to document evidence of HCV infection by HCV RNA, HCV antibody, and ALT/AST testing. There was no evidence for HCV infection in this second recipient chimp.

**Experiment II: Testing the Infectivity of Plasma During the Eclipse Phase**

In Experiment II, eclipse phase RNA positive and RNA negative donations were selected from 5 of the 37 source plasma donors who had intermittent HCV RNA detected in the eclipse phase of infection (Figure 1 and Supplementary Figure 1 panels F-J). Chimp X331, who had not been infected in phase II of Experiment I, was used for these studies, which were begun 22.5 months after the completion of Experiment I. In Phase I of experiment II, a 250 mL plasma pool from non-viremic donations obtained during the eclipse phase of infection from five different source plasma donors was infused into Chimp X331 (see Supplementary Figure 1, panels F-J, for time points used in these infusions); these HCV RNA negative donations were collected during the RNA-negative “valleys” between intermittent HCV RNA positive eclipse phase donations (blips). This chimp was monitored weekly for 6 weeks for HCV RNA, HCV antibody, ALT, and AST. There was no evidence for viremia, antibody seroconversion or transaminase elevation, indicating that these HCV RNA negative eclipse phase donations were not infectious.

One month later, Phase II of this second experiment involved infusions of 50 mL of low-level HCV RNA positive eclipse phase plasma from each of the same five source plasma donors into the same chimp (Table 1 and Supplementary Figure 1 panels F-J). The infusions of the five units were separated by 6 week intervals during which the recipient chimp was monitored for evidence of HCV infection as described above. There was no virological evidence for transmission of HCV infection, and no evidence for humoral or cellular immune responses.

Finally, after an additional 6 month interval, Phase III of this experiment was conducted. In light of the multiple exposures to HCV RNA from eclipse phase infusions, the aim of this phase was to establish that Chimp X331 was susceptible to HCV infection when infused with a sufficient HCV RNA dose. Three 50 mL plasma infusions from HCV RNA positive units obtained either immediately prior to ramp up or during the ramp-up phase of infection from one of the five
donors (plasma donor 10083) used in Phases I and II were infused at 8 week intervals (Figure 2 - panel B and Table 2). These three units had increasing HCV viral loads (1.5 copies/mL, 3.0 copies/mL and 6.8 x 10⁵ copies/mL). The chimp was monitored weekly for 8 weeks for HCV RNA, HCV antibody, and ALT/AST after the first two infusions and then weekly for 12 weeks and biweekly until week 24 after the third infusion. Liver biopsies were performed immediately prior to the infusion containing 6.8 x 10⁵ copies/mL and at weeks 2, 3, 5 and 7 after that infusion. No HCV infection was detected over an 8 week follow-up period after each of the first two infusions (infusion of 74 HCV RNA copies [1.5 copies/mL x 50 mL] and 149 HCV RNA copies [3.0 copies/mL x 50 mL]). In contrast, after infusion of the donation with 6.8 x 10⁵ HCV copies/mL (total infusion of 3.4 x 10⁶ copies [6.8 x 10⁵ copies/mL x 50 mL]), the recipient chimp developed viremia at one week post-infusion (peak titer 1.3 x 10⁵ copies/mL) which persisted until week 16 (Figure 5). All liver biopsies were normal. Unexpectedly, HCV antibody never developed over the 24 weeks of follow-up.

**Experiment III: Testing Whether Transient HCV Infection Protected Against Rechallenge**

Experiment III (Figure 1) was conducted to determine whether the acquisition of HCV infection from a low dose inoculum, followed by viral clearance, would protect against rechallenge by the same HCV strain. In this experiment, performed 34 months after recovery from the initial experimental transmission of HCV to chimp X355, this same chimp was challenged with a 50 mL plasma infusion from the same low-viremic (1.6 copies/mL), early window phase donation that had transmitted the initial HCV infection. The chimp was monitored weekly for 12 weeks during which time there was no evidence of a second HCV infection, implying some level of immunity even though anti-HCV and HCV-specific CMI were not detectable prior to or after the HCV challenge.

To further assess protective immunity, four weeks later the chimp was re-challenged with a 50 mL plasma infusion from the subsequent ramp-up phase donation (donated four days later) given by this same donor, which contained 6,000 HCV RNA copies/mL (for a total infusion of 300,000 HCV RNA copies). The chimp was monitored weekly for 10 weeks, then biweekly until week 24, and then monthly until week 52. Chimp X355 was reinfected with HCV as evidenced by
development of low-level HCV viremia that was only present at weeks 1, 2, and 3 and only quantifiable at week 1 (1620 copies/mL). HCV antibody developed at week 5 and persisted through week 20, was intermittently detected at weeks 28 and 32 and then reverted to negative on three subsequent measurements. ALT remained normal throughout the one year follow-up period and this chimp did not mount any PBMC proliferative responses to the challenge inoculum. At week 52, the chimp expired from an unrelated pyelonephritis and E. coli sepsis precluding any further follow-up.
Discussion

We studied the infectivity of human plasma collected in various stages of acute HCV infection to define the relationship between HCV RNA as detected by the most sensitive RNA assays and infectivity in the well-established chimp infection model. We demonstrated that infusions of 50 mL of plasma from each of five human source plasma donors thought to be in the late eclipse phase of infection (just prior to or at the beginning of the ramp-up phase) at the time of donation into an HCV-naïve chimpanzee resulted in a mild and transient HCV infection characterized by low-level viremia, HCV antibody production, and HCV-specific T cell immune responses. This was followed by viral clearance, rapid loss of HCV antibody, and marked diminution of the cellular immune response. Sequencing of isolates established that the infection was from a single plasma donor in the group of 5 used in the experimental infusion. Based on performing HCV TMA on 27 replicate samples from the transmitting donation, we established that this plasma had an estimated HCV RNA concentration of 1.2 copies per mL and hence the donor was probably in the early phase of HCV ramp-up viremia (rather than the eclipse phase) at the time of this donation. The definition of the transition between the eclipse and ramp-up phases is not precise and the mechanisms leading to this transition are not known, as discussed in Glynn et al. Based on the 50 mL plasma infusion from this donation, we estimated that 60 HCV RNA copies were infused into the recipient chimp. Samples collected from 3 to 14 days previously from the same donor were infused in 50 mL aliquots into another HCV naïve chimp and did not cause infection, indicating that the transmitting donation was obtained at or near the time when the donor first became infectious and that the duration of infectious viremia prior to detectable RNA by donor screening nucleic acid assay technology (NAT) assays was very brief.

This experimental transmission has some parallels with the first reported human case of transfusion-transmitted HCV infection from a unit retrospectively shown to be HCV RNA negative by a sensitive individual donation NAT (ID NAT) assay. In this human transmission, HCV RNA could be demonstrated inconsistently in the transmitting donation when evaluated in multiple replicates by dHCV TMA testing, leading to the conclusion that HCV RNA was present, but at a very low level (estimated at <10 copies/mL). The donor of this unit may have been in the very early ramp-up phase with HCV RNA levels below the level of consistent
detection by even the most sensitive ID NAT screening method, as occurred in our chimp
transmission experiment. Data from this study in chimpanzees, other chimp experimental studies
using serially diluted ramp-up phase plasma infusions,\textsuperscript{14, 16} and the clinical case report of a
breakthrough transmission\textsuperscript{29} demonstrate that ID NAT cannot completely eliminate the risk of
transfusion transmission of HCV, given the high level infectivity of ramp-up phase virus. Of
relevance to transfusion safety, it appears that blood transfusions from donors in the early stage
of acute HCV infection can be infectious prior to the time of RNA detection by routinely
performed NAT screening, including the most sensitive ID NAT test currently available which
has a 50\% limit of detection of 12.1 copies/mL (95\% CI 11.1–13.2).\textsuperscript{26} Hence, conversion to
individual donation NAT from currently employed mini-pool NAT (MP NAT) screening will not
close the infectious window period completely and may not be warranted given its projected very
low incremental yield and poor cost-effectiveness.\textsuperscript{10, 25, 30}

A further set of experiments were performed to evaluate the infectivity of two types of donations
from the surprisingly prolonged eclipse phase of infection observed in 74\% of the plasma donor
panels we studied. These included donations that were HCV RNA positive and estimated to
contain from 1 to 20 HCV RNA copies/mL (60 to 561 HCV RNA copies per infusion) and other
intervening donations from the same donors that were HCV RNA negative by sensitive multi-
replicate testing. Infusions of 50 mL of plasma from either of these two donation types from each
of five donors with such intermittent HCV RNA “blips” did not cause infection when transfused
into a single chimp, who was later proven to be susceptible to HCV infection when infused with
a ramp-up phase viremic sample from one of these same donors.

Thus, despite the fact that HCV RNA was intermittently detected during the eclipse phase of
infection for up to 8 weeks prior to ramp-up viremia, our results show that transfusion of such
units was not infectious in chimpanzees. In contrast, we demonstrated infectivity with an early
ramp-up phase sample at a total infusion dose of 60 HCV RNA copies. Japanese investigators
have shown that even lower doses of HCV RNA (total inocula of 20 HCV RNA copies) from an
acutely infected chimp transmitted HCV infection to recipient chimps.\textsuperscript{14} Hence, during the ramp-
up phase of early HCV infection, infusions with very low copy numbers are highly infectious
whereas infusions having equal or higher copy numbers of HCV RNA obtained during the
eclipse phase are not infectious. This suggests critical differences in the presentation or packaging of HCV RNA during the eclipse and ramp-up phases of HCV infection as discussed below.

There are multiple possible explanations for the finding of intermittent HCV RNA detection in some donors during the eclipse phase. This may represent persistent low-level viremia that occurs at a concentration that is at or near the limit of assay detection and thus, based on assay limitations, is detectable in some samples and non-detectable in other serially collected specimens. Another explanation is that periods of HCV viremia alternate with non-viremic periods as a result of intermittent release of viral particles into the serum from focal replication sites such as the liver and possibly even peripheral blood cells. Thirdly, these episodes could represent repeated HCV exposures or reinfections which abort, until a particularly fit variant is finally transmitted and disseminates. Perhaps the most likely explanation is that early in HCV infection, HCV RNA can be released into plasma as non-virion associated nucleic acid or as defective, non-replication competent virions. The data appear to be most consistent with the hypothesis that complete infectious particles likely do not circulate until immediately prior to the ramp-up phase of HCV infection, leaving a very narrow window of infectivity prior to detectability by sensitive blood donor screening and diagnostic assays.

Whatever the explanation, this series of experiments, and those of others,\textsuperscript{14,16} establish that HCV RNA intermittently detected during the eclipse phase of HCV infection can be ignored when performing mathematical modeling of residual transfusion-transmitted HCV risk. This lack of infectivity (or perhaps very rare infectivity, which we were not able to demonstrate in our small study) is consistent with reports of only a very few transfusion-transmissions from HCV NAT-screened units. Thus, risk modeling can employ the time period from the extrapolated beginning of ramp-up phase viremia to detectable virus by the screening method employed (ID NAT, MP NAT, 4\textsuperscript{th} generation Ag/Ab assays), as reviewed in detail elsewhere.\textsuperscript{2}

We have shown that a chimp infected with HCV from infusion of a low dose early ramp-up inoculation developed a transient HCV infection as evidenced by low-level and short-lived viremia, and both humoral and cellular immune responses; by week 17, virologic, serologic and
biochemical evidence of HCV infection had disappeared and very low-level T-cell responses were the only residua of past HCV infection. A second chimp also inoculated with plasma from the early ramp-up phase of HCV infection developed transient viremia but failed to seroconvert. Similar examples of transient infections without seroconversion or with seroconversion followed by seroreversion have been reported in experimental chimp infectivity studies, and rarely in human HCV infections following transfusion and injection drug use exposures. Our experimental findings corroborate these previously reported human observations in establishing that more HCV infections occur in persons than are clinically, virologically or serologically identified by cross-sectional serological or NAT screening.

Lastly, these experiments in a single chimp indicate that HCV infection from a low dose inoculum did not confer protective immunity to rechallenge, as we demonstrated only limited protection when the chimp who developed but then lost detectable adaptive humoral and cellular immunity was rechallenged with a higher dose of the identical HCV strain.

A noteworthy limitation of our study is that we had access to only two HCV naïve chimps to evaluate the infectivity of a large number of donations from 10 donors with different patterns of eclipse and ramp-up phase viremia. Consequently we had to judiciously design experiments with reuse of the same chimps for multiple infusion experiments. This could have resulted in either immunization or tolerance to HCV, perturbing our ability to ascertain infectivity of donor plasma. However, we do not believe this seriously compromised interpretation of our experiments, since all the chimps were carefully evaluated using sensitive assays for detection of HCV RNA and both humoral and cell mediated immunity and each animal was negative by all these parameters prior to viral challenge. Further, in the chimp (X331) that was uninfected after challenge with blip and valley eclipse phase infusions, we confirmed HCV susceptibility by transfusing a high titer inoculum from the ramp-up phase of infection.

In summary these studies demonstrate that: 1) large volume plasma transfusions can transmit HCV infection prior to RNA detection by current donor screening assays, but the interval of infectivity prior to RNA detection appears to be very brief (4 days in this experiment); 2) the non-infectivity of eclipse phase “blips” indicates that these may represent incomplete virions and
can be ignored when calculating HCV residual transfusion risk; 3) markers of HCV infection can be rapidly lost after exposure to low-dose inocula, suggesting that more HCV infections occur than are currently documented; 4) HCV infection from a low dose inoculum does not necessarily confer protective immunity to rechallenge from a higher dose of the homologous strain.
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Author Contributions


*Conduct and monitoring of animal research:* K.K. Murthy, H.J. Alter.


Author Conflicts of Interest Disclosures

The authors have no conflicts to disclose.
References


Table 1: Estimated HCV RNA concentrations of eclipse phase donations with blip viremia infused into chimp X331 (Experiment II, Phase II)

<table>
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<tr>
<th>Donor/donation #</th>
<th>Days preceding ramp-up&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Number (%) replicates TMA positive</th>
<th>Imputed HCV RNA copies/mL (95% Fiducial Limits)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Infused HCV RNA copies</th>
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<td>10050-04</td>
<td>41</td>
<td>6/27 (22%)</td>
<td>2.22 (1.23-3.17)</td>
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<tr>
<td>10083-02</td>
<td>49</td>
<td>23/27 (85%)</td>
<td>11.22 (9.35-13.67)</td>
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<td>10011-02</td>
<td>49</td>
<td>2/27 (7%)</td>
<td>1.18 (0.53-1.91)</td>
<td>60</td>
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<tr>
<td>10029-01</td>
<td>48</td>
<td>10/27 (38%)</td>
<td>3.37 (2.15-4.47)</td>
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</tr>
<tr>
<td>10085-02</td>
<td>49</td>
<td>10/27 (37%)</td>
<td>3.29 (2.08-4.38)</td>
<td>164</td>
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</tbody>
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1. Plasma from HCV RNA negative donations from each of these five donors were previously infused into chimp X331 and resulted in no evidence of HCV infection. These donations were collected prior to ramp-up viremia at days -31, -23, -19, -21, and -18 respectively.

2. RNA copy levels were estimated from probit analysis of replicate testing of the HCV WHO International Standard (NIBSC Codes: 97/690); copies/mL were based on a conversion factor of 3.4 RNA copies/International Unit (IU).
Table 2: Estimated HCV RNA concentrations of donations infused into chimp X331 to prove susceptibility to HCV infection (Experiment II, Phase III)

<table>
<thead>
<tr>
<th>Donor/donation #</th>
<th>Phase of infection</th>
<th>Days preceding ramp-up</th>
<th>Number (%) replicates TMA positive</th>
<th>HCV RNA copies/mL (imputed from replicate TMA testing(^2) or measured by VL assay)</th>
<th>Infused HCV RNA copies</th>
</tr>
</thead>
<tbody>
<tr>
<td>10083-12</td>
<td>Prior to ramp-up</td>
<td>7</td>
<td>3/27 (11%)</td>
<td>1.48 (0.71-2.28)</td>
<td>74</td>
</tr>
<tr>
<td>10083-13</td>
<td>Prior to ramp-up</td>
<td>5</td>
<td>9/27 (33%)</td>
<td>2.99 (1.83-4.05)</td>
<td>149</td>
</tr>
<tr>
<td>10083-14</td>
<td>Ramp-up</td>
<td>0</td>
<td>NA(^1)</td>
<td>6.8 x 10^5</td>
<td>3.4 x 10^6</td>
</tr>
</tbody>
</table>

1. This donation was positive by quantitative HCV RNA testing at a concentration of 6.8 x 10^5 copies/mL and was not subjected to replicate TMA testing.

2. RNA copy levels were estimated from probit analysis of replicate testing of the HCV WHO International Standard (NIBSC Codes: 97/690); copies/mL were based on a conversion factor of 3.4 RNA copies/International Unit (IU).
Figure Legends

Figure 1: Experimental design and sequence of plasma infusions and follow-up of chimpanzees.

Experiment I assessed the infectivity of plasma that tested HCV RNA negative by licensed diagnostic assays and was obtained in the days just prior to ramp-up viremia. A) 50mL of pre-ramp-up phase plasma from each of 5 commercial apheresis donors was infused sequentially during a single experimental procedure into chimp X355. B) When transmission was linked to one donor by phylogenetic sequencing, 50 mL plasma samples from each of four earlier donations from that implicated donor were transfused to a second animal (X331) at 9 week intervals. Both animals were followed for virologic, serologic and cell mediated immune responses to assess evidence of HCV infection.

Experiment II examined the infectivity of samples from 5 donors who had intermittent low level HCV RNA ("blips") detected during the eclipse phase of HCV infection by infusions into chimp X331. C) Phase I: Infusion of a pool of 250 mL (50mL plasma/donor from donations collected subsequent to blips) of HCV RNA negative plasma from the eclipse phase. D) Phase II: 50 mL plasma samples from blip viremic units from the eclipse phase from the same 5 donors were sequentially infused at 6 week intervals. Phase I and phase II infusions did not transmit HCV infection to the recipient animal (chimp X331). E) Phase III: To confirm this chimp’s susceptibility to HCV infection, 50 mL plasma samples from each of 3 progressively higher titer HCV RNA positive donations collected during the early ramp-up phase of infection from one of these 5 donors were infused at 8 week intervals.

In Experiment III, chimp x355 who had spontaneously recovered from HCV infection and lost anti-HCV as well as virus, was rechallenged three years later to determine whether prior infection conferred protection against reinfection. F1) Infusion of 50 mL plasma containing an estimated 80 HCV RNA copies (1.6 copies/mL) from the previous infecting donation; F2) Rechallenge with 50 mL plasma containing an estimated 300,000 HCV RNA copies (6,000 copies/mL) from a subsequent early ramp-up phase donation from the same donor.

HCV = hepatitis C virus.
RNA = ribonucleic acid.

Figure 2. Representative viremia results from two of the ten plasma donors used in chimpanzee infection experiments (data from all 10 panels is presented in supplementary figure 1). Panel A shows TMA and viral load results over time from plasma donor panel 10081, who did not demonstrate intermittent blips of HCV RNA prior to detection of ramp-up phase viremia. Numbers above bars represent the order in which aliquots were infused into chimps. Infusion 1 was part of a transmitting pool that was infused into chimp X355. Infusions 2-5 were each infused into chimp X331 at intervals of 9 weeks between infusions. Viral load in the ramp-up and post-ramp up phase of HCV infection in donor 10081 is indicated by black triangles. Panel B presents TMA and viral load results over time from plasma donor panel 10083 who manifested intermittent blips of HCV RNA reactivity during the eclipse phase of acute HCV infection. Both HCV RNA negative “valley” and HCV RNA positive “blip” samples were infused into chimp X331 (see Fig. 1, Experiment II). Numbers above bars identify aliquots and the order infused into chimps. Note that the units were infused in a different order than they were collected and that infusion 1 was part of a non-transmitting pool. Viral load in donor 10083 is indicated by black triangles. TMA = transcription-mediated analysis assay. HCV = hepatitis C virus. RNA = ribonucleic acid.

Figure 3: Results of transmission experiment I in chimp X355. 50 mL plasma from each of 5 donors in the pre-ramp-up phase of HCV infection were infused in a single experimental procedure into chimp X355 (see Fig. 1, Experiment I) and infectivity monitored by weekly or biweekly measurement of ALT level (solid line), HCV RNA (dashed line and box), anti-HCV (box) and cell-mediated immune response (lower panel). Transient HCV infection is demonstrated. Note that HCV RNA was no longer detectable by week 10 and that antibody to HCV disappeared by week 18 after infusion. Cell mediated immune responses, measured in a proliferation assay, peaked at week 4 and considerably diminished by week 10. HCV = hepatitis C virus. ALT = alanine aminotransferase.
RNA = ribonucleic acid.

**Figure 4: Phylogenetic reconstruction of HCV E1/E2 region sequences from 5 plasma donors (10081, 10051, 10082, 10017 and 10022) and a chimpanzee (X355) infused with a pool of 50 mL plasma from each of the donors.** Population sequences corresponding to the first hypervariable region (HVR-1) of E1/E2 (404nt) for donors and the chimp are indicated by a filled triangle (▲) and cloned sequences by an open triangle (Δ). Samples from two time points 19 days apart were obtained from the chimpanzee. The first time point sequences are indicated with c and the second time point sequences by C. Viral sequences in the chimp are shown to be closely related to donor 10081. Reference sequences from genotypes 1a (HCV-H, H77 and HCV-1), 1b (L2, JK1) and 3a (K3a) were included. The Maximum Likelihood tree was rooted with the 3a reference sequence. Bootstrap values greater than 70% are indicated.

HCV = hepatitis C virus.

**Figure 5: Results of transmission experiments in chimp 331 (See Fig. 1, Experiment II).** After demonstrating that valley phase, blip phase and pre-ramp-up phase infusions (50 ml) from each of 5 plasma donors were not infectious in chimp X331, this animal was challenged with a ramp-up phase inoculum containing 340,000 total RNA copies in a volume of 50 mL. Chimp X331 was infected as indicated by the detection of HCV RNA from weeks 1 to 16, but then HCV RNA cleared; HCV antibodies and cell mediated immune responses to HCV were not detected and the ALT level was not elevated. By week 18 there was no residual evidence that this infection had occurred.

HCV = hepatitis C virus.

RNA = ribonucleic acid.

ALT = alanine aminotransferase.
Experiment I (Infectivity of very early ramp-up plasma)

1. Plasmapheresis donors → Identification of Infectious Donor by Sequencing → HCV Naive Chimp X355
2. HCV Naive Chimp X331

Experiment II (Infectivity of eclipse phase plasma)

1. Serial donor samples → Blip → Valley → Eclipse → Pre → Ramp-up
2. Chimp X331

Experiment III (Rechallenge of transiently infected chimp)

1. Chimp X355
2. 3 years after recovery
3. 3 years after HCV clearance
4. Serial follow-up for HCV infection → Serial F/U for HCV Infection

Figure 1. Study Design
Figure 2_Representative Donor Plasma Panels

A

Simple HCV Panel 10081

Days Pre & Post HCV RNA Detection by HCV Monitor Assay

Replicates Tested by TMA

B

Blippy HCV Panel 10083

Days Pre & Post HCV RNA Detection by HCV Monitor Assay

Replicates Tested by TMA

Reactive  Non- Reactive  Viral Load (IU / mL)
Figure 3. CHALLENGE CHIMP X355
Figure 4: Phylogenetic Tree

[Diagram of a phylogenetic tree with labels and annotations indicating branch lengths and tree topology.]
Valley Phase Infusions 50ml X 5 → No Infection
Blip Phase Infusions 50ml X 5 → No Infection
Pre Ramp-up (TMA+/PCR-) 50ml X 5 → No Infection

ANTI-HCV
- - - - - - - - - - - - - - - -

HCV-RNA
- + + + + + + + + + + + + + + + + + + + + + - - - - - -

Infusion: Ramp-up phase containing 340,000 total RNA copies

Figure 5. CHALLENGE CHIMP X331
Infectivity in chimpanzees (Pan troglodytes) of plasma collected before HCV RNA detectability by FDA-licensed assays: implications for transfusion safety and HCV infection outcomes