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Wide Range of Quasispecies Diversity during Primary Hepatitis C Virus Infection

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Hepatitis C virus (HCV) infections may be initiated by multiple infectious particles, resulting in a genetically heterogeneous viral population, or by a single particle, leading to a clonal population in the initial stage of infection. To determine which of these scenarios is most common, we evaluated the genetic diversity of HCV quasispecies in 12 seronegative subjects with primary infection following community exposures, six acutely infected recipients of HCV-seropositive blood transfusions and six seropositive individuals with infections of undetermined durations. RNA isolated from plasma and a region of the HCV envelope gene including the first hypervariable region (HVR-1) was reverse transcription-PCR amplified and subcloned, and multiple plasmid clones were sequenced. Phylogenetic analysis indicated that all HCV variants clustered by individuals. Genetic distances among HCV variants within recently infected subjects ranged from 1 to 7.8%. On the basis of the estimated mutation rate of HCV in vivo and the *Taq* polymerase error rate, primary infection viral quasispecies were classified as genetically heterogeneous when the maximum sequence divergence between genetic variants in the same person was >3%. Heterogeneous quasispecies were detected in 4 of 12 preseroconversion subjects, 1 of 6 transfusion recipients, and 4 of 6 seropositive subjects. The high level of viral quasispecies genetic diversity found in at least a third of recently infected individuals is consistent with the transmission of multiple infectious particles. Community-acquired HCV infection, predominantly the result of needle sharing by injection drug users, therefore appears to be frequently initiated by the successful transmission of multiple viral variants.

Hepatitis C virus (HCV) in plasma is typically found as a genetically heterogeneous, monophyletic population of viral variants often referred to as a quasispecies (14, 18, 49). The composition of such quasispecies changes rapidly in immunocompetent hosts, presumably because of the selection of escape variants that evade cellular and humoral immune responses (9, 22, 31, 59, 68) or because of superinfection with a divergent strain (25, 55). Limited HCV evolution has been reported in immunocompromised chimpanzees and humans, possibly because of reduced immune selective pressures (6, 44, 47, 66). The high mutation rate and short generation time of HCV therefore provide this virus with a high level of genetic adaptability, which may explain why as many as 80% of primary infections result in chronic infection with high-titer viremia (43).

Because primary HCV infection is typically asymptomatic, subjects recently infected with HCV through contaminated needle sharing or other community exposures are difficult to identify. Consequently, the genetic diversity of the viral quasispecies during the very early preseroconversion phase of viremia remains largely unknown. The genetic diversity of preseroconversion plasma quasispecies is likely to be closely related to that of the inoculums since they are separated by, at most, a few weeks of evolution and may be used to test whether community-acquired HCV primary infection is a clonal event, initiated by a single infectious particle, or an oligoclonal event involving the transmission of multiple variants. Although the typical inoculum volume is low, the high HCV RNA load found in plasma suggests that needle sharing could conceivably result in the transmission of multiple particles. On the other hand, the likely presence of replication-defective and antibody-neutralized particles (39) may result in a high ratio of noninfectious to infectious particles. The need for an infectious particle to breach innate immune barriers and reach and infect a susceptible target cell may further reduce the number of infectious units initiating a new infection.

In this study, we measured viral quasispecies diversity in 12 early viremic, preseroconversion specimens from individuals with community-acquired HCV infection. We report on the transmission of multiple distinct genetic variants in a third of these cases.

MATERIALS AND METHODS

Subjects, HCV RNA testing, viral loads, and serological assays. Twelve initially anti-HCV antibody-negative frequent plasma donors (PD) with primary HCV infections were identified by pooled-specimen nucleic acid amplification testing, using the UltraQual HCV reverse transcription (RT)-PCR assay (National Genetics Institute, Santa Monica, Calif.). The UltraQual assay has a 95% detection limit of 17.1 HCV RNA copies/ml. Plasma samples from six blood transfusion recipients collected in the 1970s by the Transfusion-Transmitted Virus Study were also obtained. These recipients had been transfused with what were later determined to be seropositive and viremic blood components. Retrospective testing of stored serial pre- and posttransfusion samples from these recipients established that they became viremic posttransfusion (48). Plasma samples from six anti-HCV antibody-positive (HCV recombinant immunoblot assay 3 [RIBA-3] reactive and HCV enzyme immunoassay [EIA 3.0] positive)

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first-time blood donors were acquired from the Blood Centers of the Pacific in San Francisco. Viral loads were determined by using the Roche COBAS Amplicor HCV monitor 2.0 assay with a detection limit of 600 HCV RNA copies/ml. The serological status of each individual was determined at multiple time points with the Ortho HCV 3.0 enzyme-linked immunosorbent assay system (Ortho-Clinical Diagnostics, Raritan, N.J.). All subjects had given informed consent for donor screening (plasma and blood donors) or research follow-up and testing (Transfusion-Transmitted Virus Study); further testing of coded samples for the present study was reviewed and approved by the University of California San Francisco Committee on Human Research.

RNA extraction, amplification, and sequencing. HCV RNA was extracted with the QIAamp Viral RNA Mini Kit (QIAGEN Inc., Valencia, Calif.). Ten microliters of extracted RNA was reverse transcribed in a 20-μl cDNA reaction mixture containing 200 U of Superscript II RNase H^- reverse transcriptase (Invitrogen, Carlsbad, Calif.) and 200 ng of random oligonucleotide hexamers in accordance with the enzyme manufacturer's instructions. A 398-bp region of the E2 gene including the first hypervariable region (HVR-1) was then amplified by nested PCR. PCR mixtures (50 μl) contained 5 μl of cDNA and 10 pmol each of sense and antisense primers with 1.25 U of *Taq* DNA polymerase (Promega, Madison, Wis.), 1.5 mmol of $MgCl_2$ per liter, and 200 mmol of deoxynucleoside triphosphates per liter. Second-round reaction mixtures were initiated with 2 μl of first-round reaction mixture. PCR primers and cycling conditions for the amplification of E1/E2 have been described previously (67). Amplified products were subcloned into plasmid pCR2.1 (Invitrogen). Plasmid DNA was purified, quantified, and sequenced directly with M13 forward and reverse primers and the BigDye version 3.0 Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.). An HCV plasmid control was amplified and cloned as described above, and seven subclones were sequenced to estimate diversity due to PCR *Taq* errors.

Adequate population sampling. Resampling of the same viral variant in multiple plasmid subclones occurs if one or only a few cDNA molecules are used as input into the nested PCR (37). Insufficient sampling of the viral population can therefore result in artificially low levels of viral diversity, particularly in low-viral-load samples (13, 15). To ensure adequate sampling of the viral populations being studied, the minimum number of amplifiable cDNA target molecules was determined by serial 10-fold limiting dilution of cDNA, followed by nested PCR. Positive PCRs were seen for duplicate nested PCRs initiated with 1% of the cDNA used to initiate the subcloned PCRs. Because at least 100 copies of HCV cDNA initiated each RT-PCR, resampling of the same variants was therefore unlikely to lead to spuriously low levels of genetic diversity.

Phylogenetic analysis and pairwise genetic distances. E1/E2 sequences were aligned with ClustalX (65). Phylogenetic analysis was performed with PAUP* 4.0 (63). Maximum-likelihood (ML) phylogenetic trees were constructed by a heuristic search with the HKY model of substitution as determined by hierarchical likelihood ratio test score criteria in Modeltest 3.06 (54). A starting tree was obtained by neighbor joining, and branch swapping was performed by subpruning regrafting. Base frequency, gamma distribution, and transition/transversion ratios were determined by Modeltest 3.06. Bootstrap analysis (100 replicates) was performed on each ML tree. Within-subject pairwise genetic distances were calculated with nucleotide sequences and ML settings identical to those used for phylogenetic analysis.

Nucleotide sequence accession numbers. HCV E1/E2 gene sequences generated in this study were submitted to GenBank under accession numbers AY805751 to AY805985.

RESULTS

HCV viral load and serology during acute infections. Samples from 12 PD with primary HCV infection were selected (Fig. 1). While the mode of transmission in these PD is not known, the most common cause of community-acquired HCV is sharing of contaminated drug injection equipment. Ten of the 12 genetically analyzed samples were collected within 10 days of a prior HCV RNA-negative donation, with the remaining 2 samples collected within 20 days. Six transfusion recipients infected by contaminated blood were also selected (see Materials and Methods). Transfusion recipients were HCV seronegative immediately prior to transfusion, and each received blood from a single HCV-infected donor (48). Immediately following transfusion, all recipients were HCV antibody positive due to passive antibody transfer. Samples from transfusion recipients selected for quasispecies genetic analysis were collected between 6 and 18 days posttransfusion (average, 11 days) (Fig. 1). The longitudinal viral RNA and anti-HCV antibody profiles are shown in Fig. 1. Six plasma samples from anti-HCV-seropositive (EIA 3.0 and RIBA-3) first-time blood donors with infections of unknown duration were also selected.

RT-PCR and quasispecies population sampling. Viral RNA was extracted from each of the 24 plasma samples, and a region of the E1/E2 genes, including HVR-1, was amplified by RT-PCR. In order to minimize resampling of the same variants in multiple subclones, the number of cDNA molecules used to initiate each nested PCR was determined to be at least 100 (see Materials and Methods). The PCR products were purified and subcloned, and an average of 9.8 plasmids (range = 5 to 15) were sequenced per quasispecies.

E1/E2 sequences (400 nucleotides) belonging to genotypes 1a ($n = 16$) and 1b ($n = 8$) were found (Fig. 2). HCV variants from each individual clustered independently with significant bootstrap support, indicating no sample mix-up, PCR contamination, or dual infections (Fig. 2).

De novo mutations and *Taq* polymerase errors. The genetic diversity seen in primary infection quasispecies may be due to the transmission of multiple variants, mutations acquired postinfection, or *Taq* polymerase errors introduced during RT-PCR or subcloning. The level of genetic diversity expected to evolve in vivo following primary infection can be approximated on the basis of previously determined mutation rates for the E1/E2 region, which ranged from 1.5×10^{-3} to 8.6×10^{-2} nucleotide substitutions per site per year (2, 3, 6, 10, 16, 29, 60). Assuming the highest reported mutation rate (calculated for the 81 nucleotides of HVR-1) (6) and a 20-day period of viral replication prior to sample collection, an upper bound estimate of pairwise genetic distance of 0.47% could be attributed to de novo mutations occurring following transmission.

To determine the contribution of *Taq* polymerase errors to the level of genetic diversity, a nested PCR was initiated with 1 to 10 copies of an HCV plasmid and seven subcloned plasmids were sequenced. Their average pairwise genetic distance was 1.0% (ranging from 0.5 to 2.1%) (Table 1), within the expected range of *Taq* errors for a nested PCR protocol (41, 62). Adding the maximum *Taq* mutation rate measured for our nested PCR (2.1%) to the expected number of de novo mutations (0.47%, assuming the fastest reported rate of substitutions over a period of 20 days), we derived a maximum background noise value of 2.6%. We therefore conservatively estimated that a primary infection quasispecies consisting of genetic variants with a maximum pairwise distance of greater than 3% reflected infection with an oligoclonal viral population.

Early quasispecies diversity. The maximum pairwise distances within preseroconversion quasispecies ranged from 1.0 to 7.8%, reflecting a wide range of quasispecies diversity during primary infection (Table 1). Average pairwise distances within these subjects ranged from 0.45 to 3.91% (Table 1). Seropositive subjects showed maximum genetic distances higher than those of acutely infected PD (t test $P = 0.045$), while average genetic distances were only marginally higher in seropositive subjects (t test $P = 0.11$).

According to the 3% maximum diversity cutoff criterion,

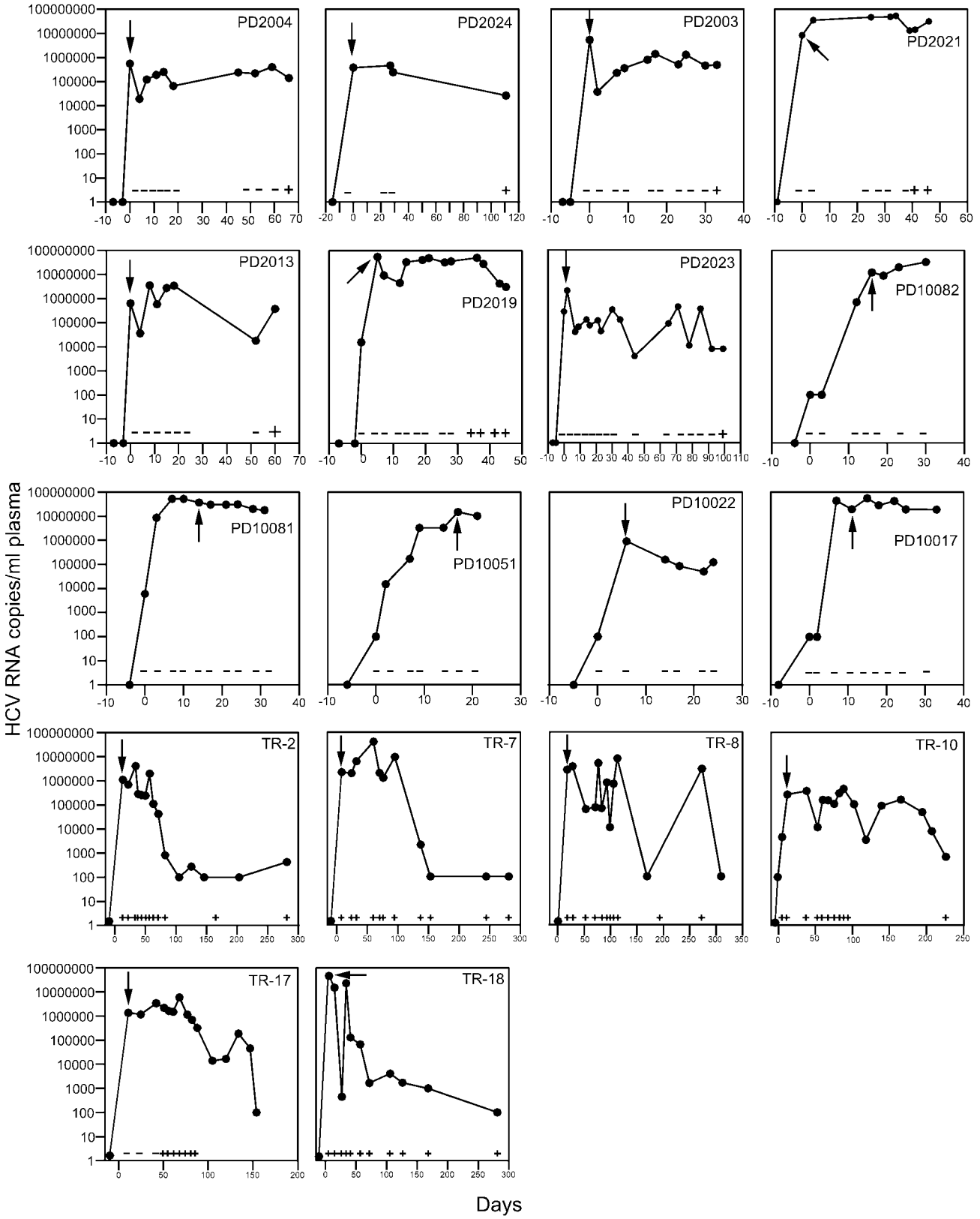

FIG. 1. Primary HCV infection: viral load measurements and HCV serology of frequent PD and transfusion recipients (TR). Anti-HCV EIA results are indicated by plus and minus signs. All transfusion recipients were EIA negative on the day of transfusion. Day 0 corresponds to the first HCV RNA-positive sample for PD and the day of transfusion for TR. Arrows indicate samples used in viral genetic analysis.

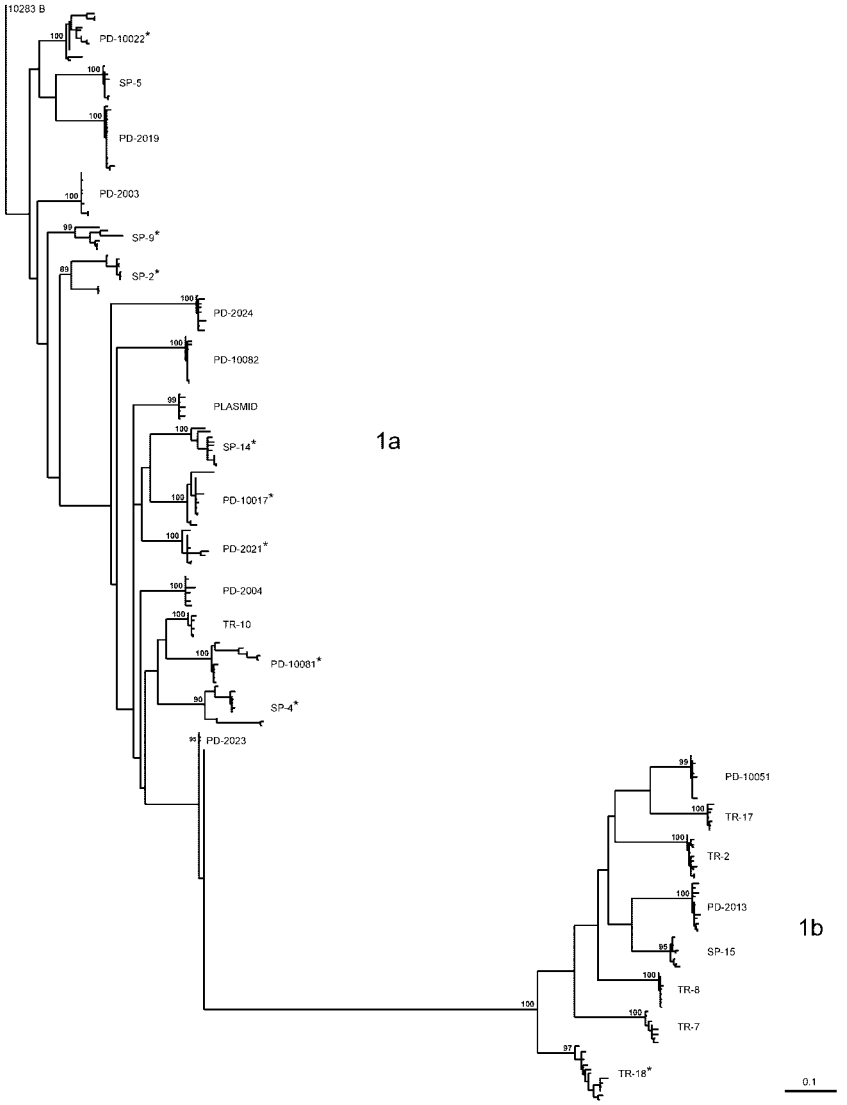

FIG. 2. Phylogenetic analysis of HCV E1/E2 sequences by ML. Transfusion recipients and seropositive subjects identified as TR and SP, respectively. Bootstrap values of greater than 70% are indicated at the nodes of branches. Individuals who have a genetically heterogeneous quasispecies (>3% maximum genetic distance) are indicated by asterisks.

homogeneous quasispecies were therefore detected in 8 of 12 subjects with community-acquired primary HCV infections while the other 4 subjects carried heterogenous quasispecies descended from more than a single transmitted variant. One in six transfusion recipients and four in six seropositive subjects with infections of unknown duration showed genetically heterogeneous quasispecies.

DISCUSSION

Following the introduction of HCV antibody and RNA testing in blood banking, HCV transmission is now largely the result of sharing contaminated equipment during injection drug use (IDU) and sexual transmission is considered to play only a minor role (7, 8, 69). HCV quasispecies diversity has been extensively studied in chronically infected humans, experimentally infected chimpanzees, human immunodeficiency virus (HIV)-coinfected subjects, and individuals on therapy and following blood product transfusions and liver transplantations (4, 5, 12, 16, 19, 23, 27, 31, 32, 50, 52, 53, 57, 58, 66, 67). A strong CTL response targeting multiple epitopes has been correlated with plasma viremia resolution (11, 34, 36, 42). Little information exists on the genetic diversity of early HCV quasispecies immediately following IDU-mediated infection. Multiple HCV variants were detected at early time points in recipients of contaminated blood transfusions and in individuals who acquired HCV either through a conjunctival blood splash or during surgical intervention (19, 33, 40). Perinatal transmission seems to restrict the diversity of the quasispecies found in the mother (30, 45), although multiple variants were observed in newborns coinfected with HIV (51). Blood transfusion recipients carried diverse quasispecies soon after infection (19). With a gel shift-based method (heteroduplex analysis plus single-stranded conformational polymorphism) and samples col-

TABLE 1. Genetic distances between HCV variants within each subject[a]

Group and subject ID[b] no.	No. of clones sequenced	% Avg intraindividual genetic distance	% Maximum intraindividual genetic distance
Acute infection			
2003	11	0.56	1.53
2013	12	1.20	2.32
2023	5	0.51	1.01
2004	8	1.12	2.56
10082	12	0.41	1.53
10051	11	0.46	1.53
2019	16	0.56	2.04
2024	9	1.25	2.31
10022	**11**	**3.91**	**7.2**
2021	**9**	**2.11**	**5.0**
10017	**13**	**1.75**	**7.83**
10081	**10**	**3.85**	**5.22**
Transfusion recipients			
10	7	0.77	1.27
17	7	1.07	1.79
18	**13**	**2.51**	**5.03**
2	10	2.51	2.05
7	8	1.68	2.57
8	9	0.45	0.76
Seropositive			
15	9	1.51	3.0
5	10	0.64	1.5
14	**6**	**2.97**	**6.1**
9	**11**	**5.44**	**8.5**
2	**7**	**2.06**	**9.2**
4	**10**	**3.16**	**9.4**
Plasmid	7	1.01	2.1

[a] Subjects infected with genetically heterogeneous quasispecies are in bold.
[b] ID, identification.

lected every 6 months from an IDU cohort, a lower level of HCV genetic diversity was reported in the initial seropositive than in later samples (67). Similar studies with plasma samples collected within 3 months of the onset of HCV viremia showed the presence of viral genetic diversity (38).

We describe here HCV sequence quasispecies diversity during the very early stages of primary infection prior to seroconversion. We found that the majority of these subjects (8 of 12) had a genetically homogenous viral population at this time point and therefore showed no evidence of having been infected by more than a single infectious particle. However, a third of the subjects (4 of 12) carried heterogeneous quasispecies with a level of genetic diversity that was too high to have been acquired in the few weeks since infection; these cases therefore likely resulted from the transmission of multiple infectious particles. Surprisingly, only one of the six subjects recently transfused with contaminated blood components (i.e., a very large inoculum) carried heterogeneous quasispecies. The low frequency of transfused subjects with heterogeneous quasispecies was unexpected considering the large number of infectious particles likely transfused. Since the source transfusions in these cases were from seropositive donors, a proportion of the diverse strains in the inoculum may have been complexed by antibody such that there was selective transmission of escape variants present in the donor. Alternatively, a phenomenon of genetic homogenization may have occurred, as has been postulated for HIV infection (35), whereby an initially diverse viral population experienced a rapid decline in apparent diversity because of the outgrowth of the fittest variant.

The proportion of oligoclonal community-acquired HCV infections we report (one of three) is likely to be an underestimate. Because of the limited number of subclones sequenced, the possibility of rapid quasispecies homogenization immediately following infection and since some of the infecting inocula themselves may have been genetically homogeneous (and therefore multiple but indistinguishable variants may have been transmitted), the number of oligoclonal community-acquired infections may be greater than one-third. A model of HCV evolution consistent with the transmission of oligoclonal quasispecies has been recently proposed after a very low level of sequence changes was seen in chimpanzees infected with a molecular clone whereby the majority of sequence changes seen in natural infections arise from selection of preexisting variants rather than de novo mutation (21).

The clinical consequences of infection with multiple HCV variants are currently unknown but could conceivably provide HCV with greater evolutionary flexibility to escape immune responses. A lower level of viral genetic diversity in subjects who cleared their viremia versus those who established a chronic infection has been reported (58). While therapeutic intervention strongly affects the variant composition of HCV quasispecies, contradictory results have appeared regarding the influences of viral genetic diversity per se on the therapeutic response rate (1, 17, 20, 24, 26, 53, 56, 61, 64, 70). The higher therapeutic response rate of recently infected versus long-term-infected patients (28, 46) could conceivably be related to the generally less genetically diverse quasispecies expected early in infection.

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