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Effect of Ribavirin and Amantadine on Early Hepatitis C Virus RNA Rebound and Clearance in Serum During Daily High-Dose Interferon

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The early rebound in serum HCV RNA during HCV dynamic studies with high-dose interferon may be due to *de novo* infection with interferon escape quasispecies. We simultaneously measured serum alanine aminotransferase (ALT) and HCV RNA at rapid intervals in chronic HCV liver disease patients during interferon therapy alone or in combination with ribavirin and amantadine. HCV RNA declined rapidly between 0 and 48 hr in all patients (phase 1). Ribavirin and amantadine significantly increased this phase 1 decline. In all four monotherapy patients with viral rebound, the increasing levels of HCV RNA were associated with a parallel increase in serum ALT, consistent with a hepatitis flare or *de novo* infection. By contrast, in the four monotherapy patients without viral rebound, and all eight patients receiving combination therapy, the slow progressive phase two decay was associated with declining serum ALT levels. Ribavirin or ribavirin and amantadine significantly and incrementally increased the phase two HCV RNA clearance. Dynamic sequencing in the HVR1 region in one rebound patient confirmed the potential for rapid evolutionary changes during interferon therapy. These preliminary data suggest that early viral rebound might be associated with *de novo* infection with interferon escape HCV variants, which in turn are attenuated by ribavirin and amantadine.

KEY WORDS: hepatitis C viral dynamics; interferon; ribavirin; amantadine; HCV clearance; HCV molecular evolutionary genetics.

Mathematical modeling of changes in plasma viral load after administration of antiviral compounds (1), has provided significant insights into the kinetics of host–virus interactions and the action of drugs used in the treatment of chronic human immunodeficiency virus, HIV (2, 3), hepatitis B virus, HBV (4), and hepatitis C virus (HCV) infections (5–9). The simple model of chronic viral infection on which mathematical models for establishing HCV dynamic parameters are based assumes the rapid initial decline in plasma HCV RNA levels (phase 1) that consistently follows high-dose daily interferon monotherapy reflects rapid inhibition of viral replication or release and that the variable and much slower phase 2 decay can be attributed to a progressive loss of infected cells (5). However, in a significant number of patients, an early rebound in serum HCV RNA that is not predicted by this deterministic simple model follows the phase 1 decline, replacing the expected phase 2 decay (5, 8, 10, 11). The model assumes constant growth and other virus–host characteristics during interferon treatment. However, HCV exists as a complex mixture of quasispecies with marked heterogeneity. As has been observed during nevirapine monotherapy for HIV (3), early emergence of drug-tolerant quasispecies that are capable of continuing *de novo* hepatocyte

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infection and replication could explain the HCV rebound. In that event, a parallel increase in serum ALT, as well as structural or molecular evolutionary changes in HCV quasispecies might be expected to accompany the rebound. Combining interferon with other antivirals should inhibit the emergence of these variants, attenuating the rebound and increasing the estimated serum clearance rates of HCV RNA.

In this study, we provide evidence for the first time that a parallel increase in the serum ALT levels accompanies HCV RNA rebound, consistent with *de novo* hepatocyte infection. We also demonstrate that combining interferon with other antivirals eliminates the rebound, and increases both phases of serum HCV RNA clearance, consistent with attenuation of escape variants. Finally, we present preliminary data from a single patient study that treatment with interferon is associated with rapid evolution and structural change within the HCV genome.

MATERIALS AND METHODS

Patient Population. The diagnosis of chronic hepatitis C virus infection was based on the simultaneous presence of anti-HCV and HCV RNA in serum. All patients were hepatitis B surface antigen negative and had been tested for HIV infection. None had received previous treatment with interferon, ribavirin, or amantadine. All but two of the patients were infected with genotype 1 hepatitis C virus.

Patients with other forms of liver disease and those actively abusing alcohol and intravenous drugs were excluded. None of the serum samples collected during the study contained significant amounts of ethanol.

For clinical diagnostic staging of the fibrosis scores, a routine pretreatment liver biopsy was performed within six months prior to the study and assessed by a single pathologist. Informed consent was obtained from all patients prior to entry into the study, which was approved by the Human Studies Subcommittee at the Veterans Administration Medical Center, Northport, New York, USA.

Study Design. Patients were hospitalized during the first two days of the study. The weights of the patients were recorded on admission. Venous blood samples, 5–10 ml, were collected 1 hr before and then at 0, 2, 4, 6, 8, 12, 16, 20, 24, 32, and 48 hr and then daily for up to 14 days, after the first injection of 10 million units subcutaneously of interferon-α-2a (IFN-α-2a). In a second group of patients assigned to receive dual combination therapy, the first morning dose of ribavirin, 400 mg, was given orally at the same time as the initial interferon injection. Ribavirin was repeated orally, 600 mg in the evening. In a third group of patients assigned to triple therapy, amantadine, 100 mg orally, was given twice daily in combination with interferon and ribavirin. The same dosing regimen for all three drugs was continued daily for the full 14 days of the study.

All blood samples were kept on ice, centrifuged, and separated within 6 hr of collection. Sera were frozen in aliquots at -20°C initially, and transferred within seven days to -80°C for storage. HCV RNA assays were performed within two weeks of sample collection.

Flu-like symptoms induced by interferon were treated with acetaminophen, 500–650 mg orally, 1 hr before interferon and repeated at a dose of 325–500 mg every 4–6 hr as needed.

The physician instructed patients on the technique of self-administration of interferon. After discharge, the patients returned to the hospital for daily collection of blood samples until day 14. To ensure compliance, they were asked to return empty vials, unused drugs, and used needles and syringes.

At the conclusion of this 14-day study period, patients were transferred to a standard regimen of 3 million units of interferon, three times weekly. Dual- and triple-combination therapy patients also continued with daily ribavirin, and ribavirin plus amantadine, respectively. They were evaluated at the end of three months for the presence or absence of HCV RNA in serum. Ribavirin with or without amantadine, was added to the treatment regimen of all monotherapy patients who had failed to clear HCV RNA in serum at three months.

Materials. Roche Pharmaceutical (Nutley, New Jersey, USA) kindly provided IFN-α-2a. Ribavirin was obtained from Schering Pharmaceutical (Kenilworth, New Jersey, USA) and amantadine from Endo Pharmaceuticals (Chadds Ford, Pennsylvania, USA).

The measurements of HCV RNA were performed using either the branched DNA, (bDNA) assay (Chiron, Emeryville, California, USA) for patients with high pretreatment copy numbers of HCV RNA or the COBAS Amplicor (Roche Diagnostics Systems, Branchburg, New Jersey, USA) for patients with low pretreatment copy numbers. Samples from individual patients were assayed in the same batch to avoid batch to batch variation. Using conversion tables for bDNA (1 IU = 6.3 eq) and for qPCR (100 copies = 60 IU) provided by the manufacturers, the results from either assay were recorded in international units (IU) per milliliter. In preliminary studies, we assayed eight serum samples using both techniques and recorded the results. The results varied by 11.4% $\pm$ 12.2 (mean $\pm$ SE).

Serum liver function tests (LFTs) were measured through the routine chemistry laboratory. All the serum samples from each individual patient were assayed in the same batch to avoid significant interassay variation. HCV genotyping was performed using a DNA line probe assay InnoLiPA (Innogenetics Inc., Norcross, Georgia, USA). Serum ethanol levels were assayed using a blood alcohol kit (Sigma Diagnostics, St. Louis, Missouri, USA).

Evaluation of Changes in E2 HVR1: Sequencing and Cloning PCR Products. We used sera obtained from patient 1 at 0, h 12, and 24 hr and days 5 and 10. We prepared PCR products from the HVR1 region according to the methods described by Gonzalez-Peralta et al (12), using eLONGASE, a high-fidelity polymerase with low error rates. Following incubation with Taq polymerase (0.7–1 units) at 72°C for 8–10 min, the PCR products were purified using Qiagen columns. This purification step was found to improve cloning efficiency. The purified PCR products were cloned into the plasmid vector pCR4-TOPO and transformed into TOP10 *E. coli* cells using the TOPO TA Cloning kit for Sequencing, version E (Invitrogen Corporation, Carlsbad, California, USA) according to the manufacturers protocol. Transformants were grown on LB plates containing either 100 μg/ml ampicillin or 50 μg/ml kanamycin, which typically yielded hundreds of colonies.

Up to 10 colonies per time point were selected at random and grown overnight in 2 ml LB or SOB medium in 15 ml loosely capped tubes at 37°C, containing 50 μg/ml ampicillin

or kanamycin. Plasmid DNA were isolated and purified using the SNAP miniprep kit, version E (Invitrogen) according to the manufacturers instructions. The concentration of the DNA were determined using a DNA Dipstick kit (Invitrogen).

DNA (500 ng) and universal T3 primers (3.2 pmol) in a total volume of 12 μl were provided to GeneWiz, a commercial laboratory for sequencing using the ABI PRISM model 373.

The nucleotide sequences from the clones were aligned by using Clustal W (1.8; EMBL European Bioinformatics Institute). The 196-bp HVR1 sequence is readily identified within the 3957-bp pCR4-TOPO plasmid, bordered by the known plasmid sequences at the PCR product insertion site.

Data Analyses and Statistical Methods. For quantitative analysis of observed changes in serum viral load after interferon therapy, we followed the model and equations described by Neumann et al (5), in which V_0 is the initial steady-state viral load; ε is the efficacy of drug treatment in reducing viral production and release, and ranges from zero (no effect) to one (complete removal of the virus in the steady state); c is the clearance rate constant and directly reflects the initial phase 1 decay slope; and t_0 is the initial delay that elapses before interferon can express its therapeutic action. Additionally, δ is the death rate constant for infected hepatocytes and directly reflects the phase 2 decay slope.

The rate of serum ALT increase or decrease, associated with the HCV rebound or decay, was calculated by a linear least-squares fit of a portion of the ALT data. In this linear fit we have considered only those ALT data points that were obtained after the nadir or point of inflection on the HCV RNA curve. These data points are represented by the filled circles in Figure 4 below.

All linear regression analyses were performed using the software program SigmaPlot 2.0 (SPSS, Chicago, Illinois, USA; www.spss.com). All nonlinear regression analyses were performed by using the computer software ViraFit 1.05 (Biokin Ltd., Pullman, Washington, USA; www.biokin.com). Statistical comparison between groups was performed by the Student's *t* test or by ANOVA.

RESULTS

Patient Demographics. Sixteen male patients were enrolled and completed the study. Details regarding age, genotype and degree of histologically assessed fibrosis (13) are shown in Table 1. All but two of the patients were infected with HCV genotype 1. One patient was positive for HIV. There were three African-Americans in the study. Two were genotype 1 and received monotherapy. A third was infected with genotype 3a and received triple therapy.

Pretreatment serum HCV RNA levels varied by less than one logarithmic unit, indicating steady state conditions.

TABLE 1. HCV RNA KINETICS AND ALT KINETICS DURING TREATMENT WITH INTERFERON, INTERFERON PLUS RIBAVIRIN (DUAL THERAPY B) AND INTERFERON PLUS RIBAVIRIN AND AMANTADINE (TRIPLE THERAPY, C)*

Regimen	*Patient*	*Age (yr)*	*Genotype*	H_x	t_0 *(hr)*	V_0	ε	c *1/hr*	r_v	δ *(1/day)*	r_{ALT} *(IU/day)*
A_R	1	59	1	F4	7.79	1.04	0.90	0.42	0.12	−(c)	+6.62
A_R	2(a, b)	40	1	F1	6.00	7.55	0.74	0.13	0.37	−(c)	+1.83
A_R	3(a)	48	1a	F4	8.33	2.17	0.75	0.22	0.19	−(c)	+0.86
A_R	4	47	1a	F0	7.08	7.37	0.91	0.34	1.08	−(c)	+1.14
A_{NR}	5	43	1b	F3	9.38	3.96	0.99	0.18	0.26	0.06	−1.01
A_{NR}	6	43	1	F0	7.89	0.25	0.99	0.46	0.04	−(d)	−2.01
A_{NR}	7	40	1	F1	5.22	4.07	0.89	0.21	0.31	0.03	−5.39
A_{NR}	8	45	1	F3	5.64	0.20	0.99	0.31	0.03	0.05	−0.73
A	*Mean ± SE*				*7.17 ± 1.45*	*3.33 ± 2.95*	*0.90 ± 0.10*	*0.28 ± 0.12*	*0.30 ± 0.34*	*0.05 ± 0.02*	
B	9	53	1b	F0	7.55	1.46	0.81	0.55	0.28	0.10	−0.52
B	10	52	1a	F1	8.0(e)	3.47	0.99	0.81	0.69	0.22	−1.12
B	11	50	1	F3	7.29	5.10	0.95	0.60	1.29	0.12	−0.70
B	12	51	2b	F0	4.48	2.54	0.99	0.29	0.41	0.20	−0.47
B	13	48	1b	F4	12.0	7.95	0.91	0.23	0.79	0.11	−3.97
B	*Mean ± SE*				*7.87 ± 2.69*	*4.10 ± 2.53*	*0.93 ± 0.08*	*0.50 ± 0.24*	*0.69 ± 0.39*	*0.15 ± 0.06*	
P	A vs B				*0.55*	*0.64*	*0.58*	*0.04*	*0.08*	*0.03*	
C	14(a)		3a		11.38	1.2	0.9	0.7	0.4	0.48	−0.06
C	15		1		12	4	0.9	0.9	1.4	0.24	−0.11
C	16		1		1.5	0.08	0.93	0.13	0.004	0.72	−0.1
C.	*Mean ± SE*				*8.29 ± 3.4*	*1.76 ± 1.17*	*0.91 ± 0.01*	*0.6 ± 0.2*	*0.6 ± 0.4*	*0.48 ± 0.11*	

*Explanation of symbols and corresponding units: t_0 (hours), delay time; V_0 ($10^6 \times$IU/ml), initial viral load; ε, efficacy; c (per hour), rate constant for viral clearance during phase 1 of therapy (0–48 hours); r_v ($10^{12} \times$virions/day), virion production rate; δ (per day), rate constant for death of infected cells during phase 2 of therapy (2–14 days); r_{ALT} (IU/day), daily rate of ALT level changes (increase or decrease) during Phase 2; P, probability (0–1, computed from Student's t statistic) that the mean values of each parameter are identical for treatment regimens. Parameters t_0, V_0, ε and c were obtained by fitting HCV data in Figure 1a, 1b to equation 1. Virion production rate r_v was computed as $V_0 \times c$, defined in equation 1, times a factor, based on body weight, corresponding to the extracellular fluid volume. The infected cell death rate constant δ is determined by nonlinear least squares fit of phase 1 + 2 data (Figure 2a,b) to equation 2. The rate of ALT changes was computed by linear regression of phase 2 data shown in Figure 3a,b. A_R = interferon monotherapy patients with rebound; A_{NR} = interferon monotherapy patients without rebound. (a), African-American; (b), HIV-positive; (c), rebound; (d), below detection limit during phase 2 (2–14 days); (e), fixed in nonlinear regression; r_v, units are 10^{12} per day; V_0 units are 10^6 IU/ml.

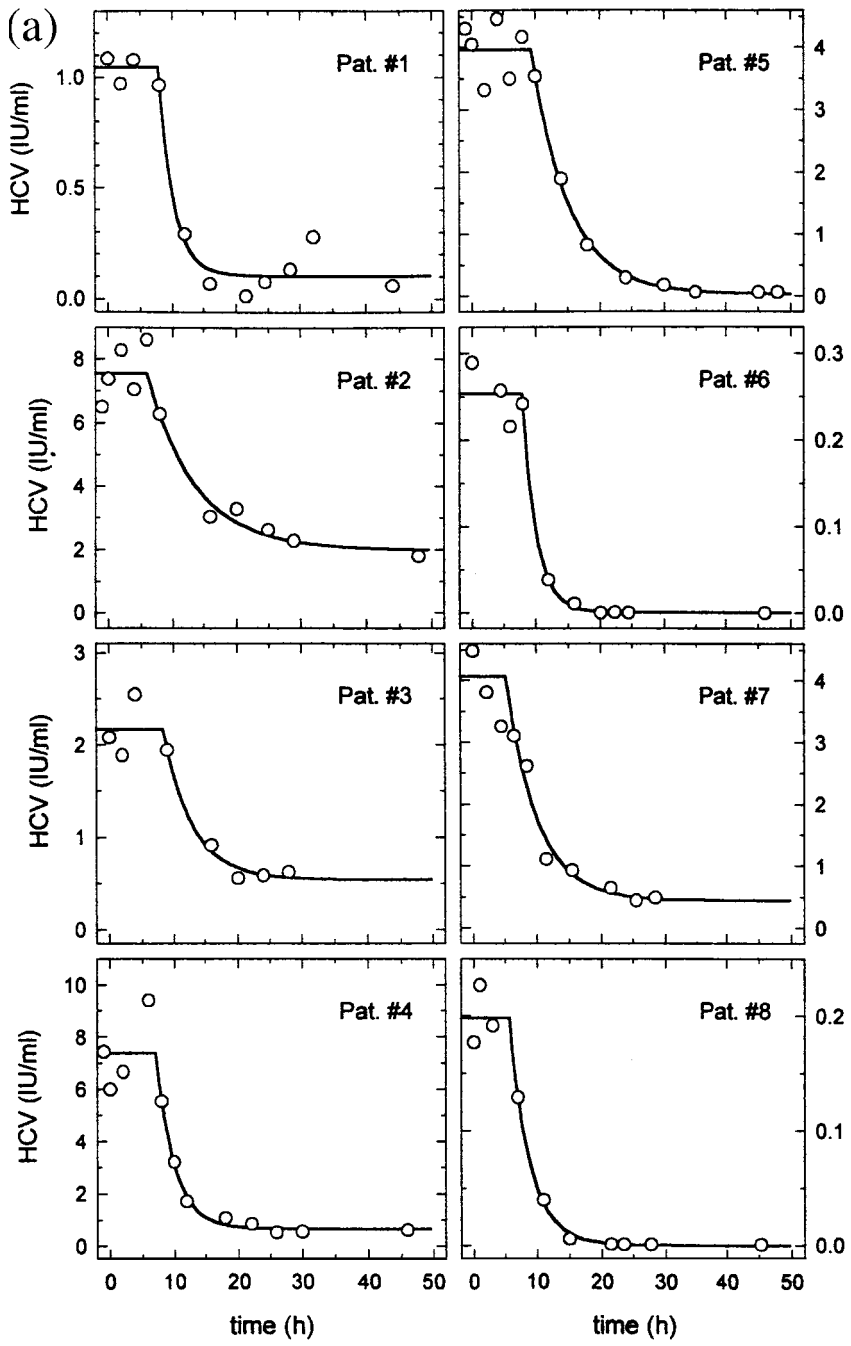

Fig 1. Changes in the HCV viral load (10^6 IU/ml) for (a) patients treated with interferon alone and (b) patients treated by the interferon plus ribavirin combination therapy during phase 1 (0–48 hr) of therapy. The solid curves were obtained by nonlinear least-squares fit to equation 1.

HCV Viral Dynamics and Serum ALT Kinetics. HCV RNA viral dynamic data observed during phase 1 (0–48 hr) for monotherapy patients and patients treated with interferon and ribavirin are shown graphically in Figure 1a, b. The data were fit to the Neumann equation in order to obtain estimates of the delay time t_0, the initial viral load V_0, the efficacy ε, and the clearance rate constant c for each patient. The results, including those from patients on triple therapy with interferon, ribavirin, and amantadine, are summarized in appropriate columns of Table 1, along with P values computed from Student's t statistic for the

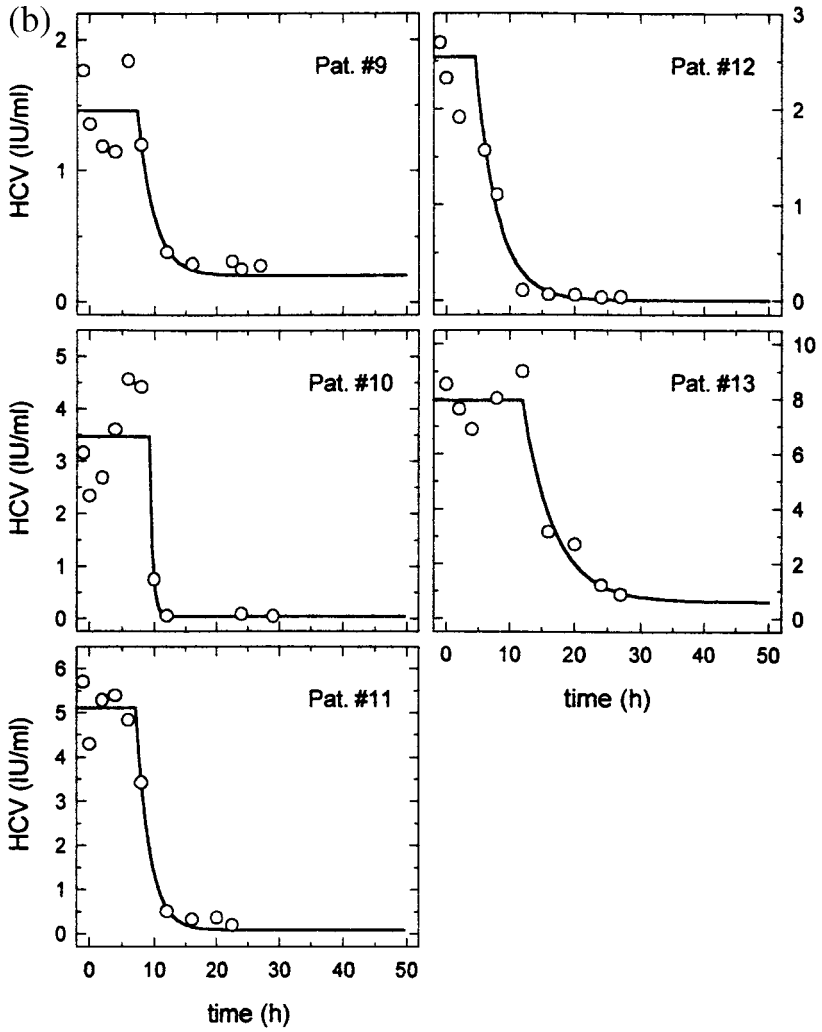

Fig 1. (Continued).

treatment. The changes in serum ALT levels during phase 1 of therapy (0–48 hr) followed no consistent pattern.

Phase 2 viral dynamic data (2–14 days) for interferon monotherapy and, dual and triple therapy are shown in Figures 2a,b and 3, 4 respectively. In four of eight patients (patients 1–4) who received interferon monotherapy, the rapid initial decline during phase 1 was followed by a marked increase or rebound in HCV RNA to levels significantly above the nadir. This rebound typically started around 24–48 hr after the initial injection, with HCV RNA levels remaining over twice the nadir at day 14. No HCV rebound was observed in the other four patients on monotherapy (patients 5–8). Although there were significant oscillations above the baseline in patient 7, serum HCV RNA by day 14 was only marginally (less than double) greater than the nadir. No significant HCV rebound was observed in any of the patients on combination therapy.

Serum ALT rose in parallel with the rise in HCV RNA in the rebound patients (Figure 4a,b), as signified by the positive slope of r_{ALT} in Table 1. By contrast, serum ALT consistently decreased in parallel with declining levels of HCV RNA in both the nonrebound patients on monotherapy and in all of the patients on combination treatment. In all of these patients, r_{ALT} was negative.

The phase 2 data were fit to the Neumann equation in order to determine the infected cell death rate constant, δ, from the slope of the decay. Since the derived equation anticipates progressively decreasing levels of HCV RNA, it was not possible to fit the data from any of the four patients on monotherapy with rebounding HCV RNA levels to the curve. In patinet 6 on monotherapy, HCV RNA levels during phase 2 were below detection limit. Thus,

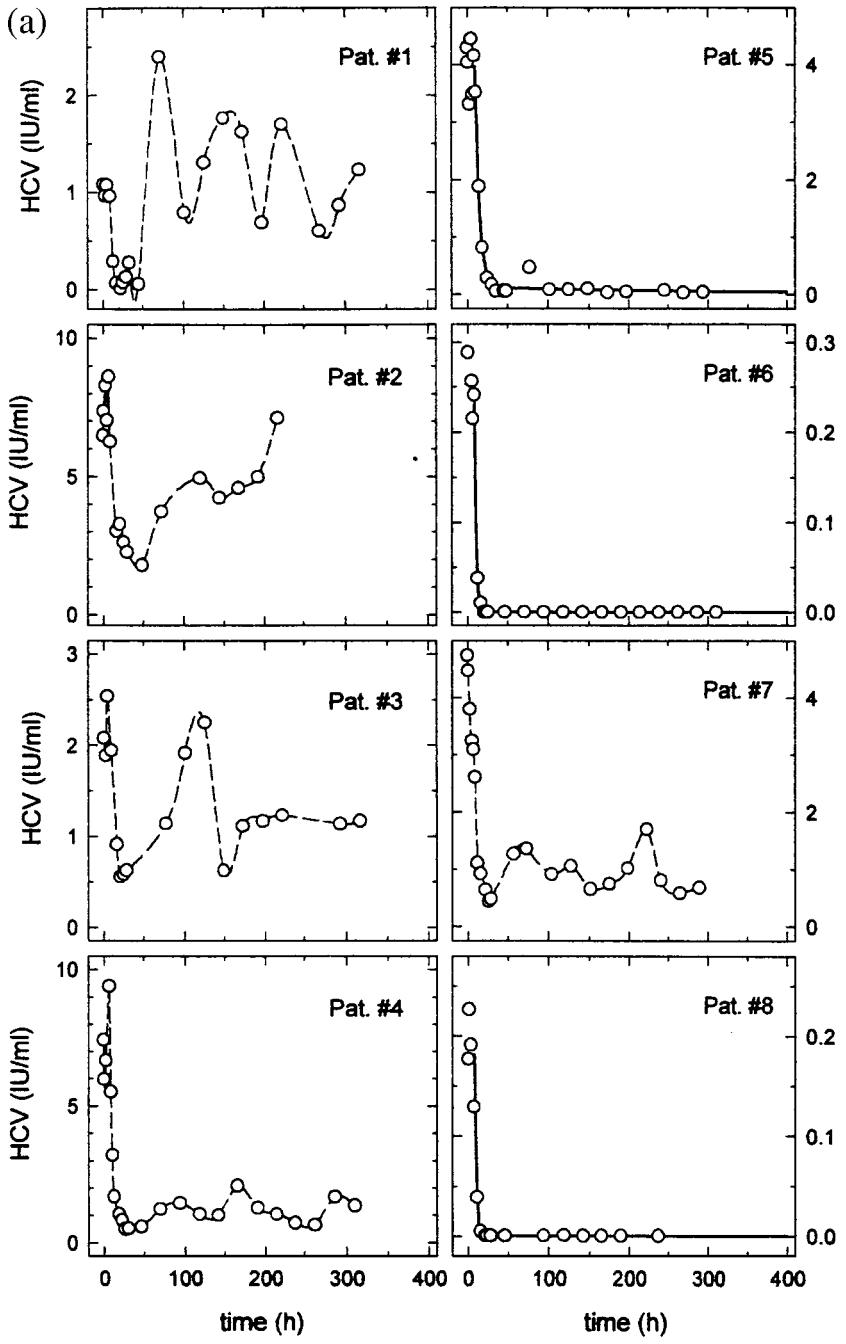

Fig 2. Changes in the HCV viral load (10^6 IU/ml) for (a) patients treated with interferon alone and (b) patients treated by the interferon plus ribavirin combination therapy during phase 2 (2–14 days) of therapy. The dashed curves are interpolated from the data for those patients where the phase 2 viral dynamics included a rebound or oscillations. The solid curves were obtained by nonlinear least-squares fit to equation 2. Data points identified by triangles were excluded from the analysis.

the infected cell death rate constant, δ, could not be determined in these five patients.

The HCV dynamic parameters for the interferon monotherapy patients (regimen A), which include the initial viral load V_0, the delay time t_0, the mean clearance rate constant c, and the daily virus production rates r_v, are similar for both patients demonstrating a rebound (A_R) and those without a significant rebound (A_{NR}). The rebound

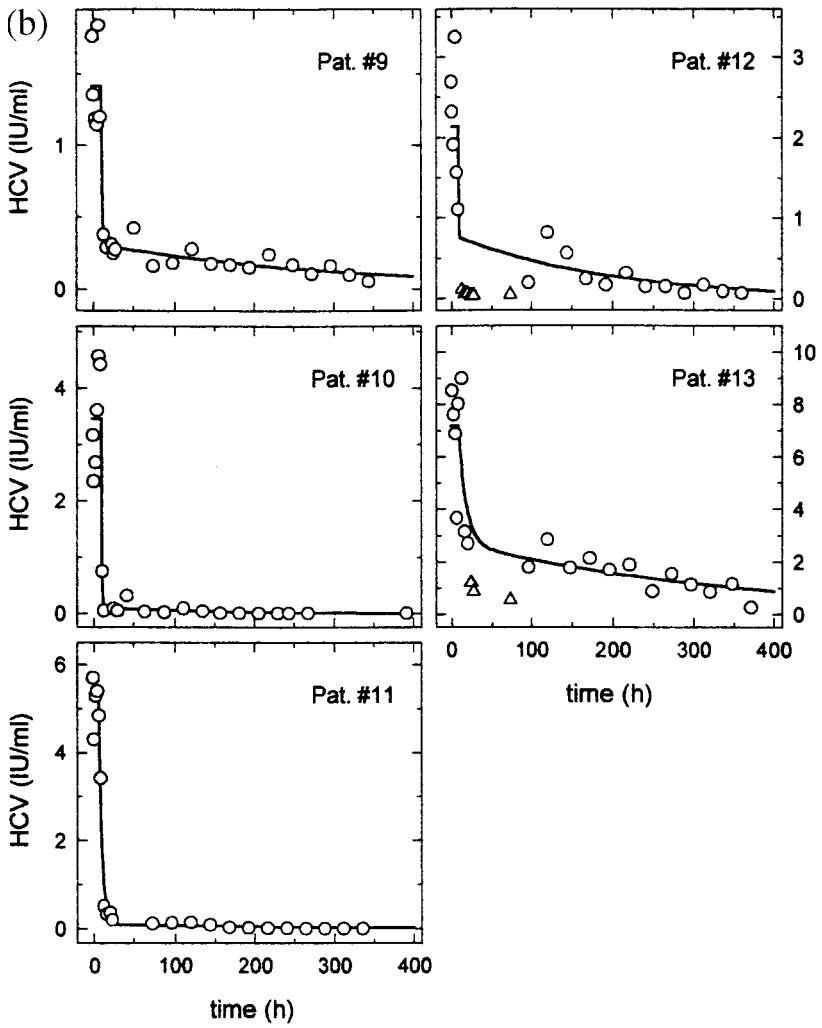

Fig 2. (Continued).

monotherapy group included two African American (AA) patients with genotype 1 HCV infection. One was also coinfected with HIV. These two AA patients had the lowest efficacies estimated in any of the groups, 0.74 and 0.75 (Table 1).

The addition of ribavirin and amantadine to interferon made no significant difference to the overall efficacy. This is consistent with their known weak antiviral activity towards HCV. By contrast, combination therapy (groups B and C combined) significantly increased the phase 1 slope and the derived estimate for viral clearance, c. The results are significant even when patients 12 (genotype 2b) and 14 (genotype 3a) are excluded from the analysis. The difference between combination group B (0.5 ± 0.24) and group C (0.6 ± 0.2) did not reach statistically significant levels, presumably because of the small numbers.

Combination therapy (groups B and C combined) also significantly increased the phase 2 slope and the derived estimate for the infected cell death rate constant, δ. These results are significant even after patients 12 (genotype 2b) and 14 (genotype 3a) have been excluded from the analysis. The combination of interferon with ribavirin and amantadine increased the calculated infected cell death rate constnat δ, almost 10-fold above that of interferon.

In almost all of the patients treated with combination interferon plus ribavirin therapy, we observed a transient increase in serum HCV RNA levels around 50–200 hr after initiation of therapy (Figure 2b). The hump was attenuated and occurred earlier, between 24 and 125 hr, in patients on amantadine (Figure 3).

Preliminary Virological Response at 3 Months. A preliminary assessment of virological response to treatment was made based on the presence or absence of HCV

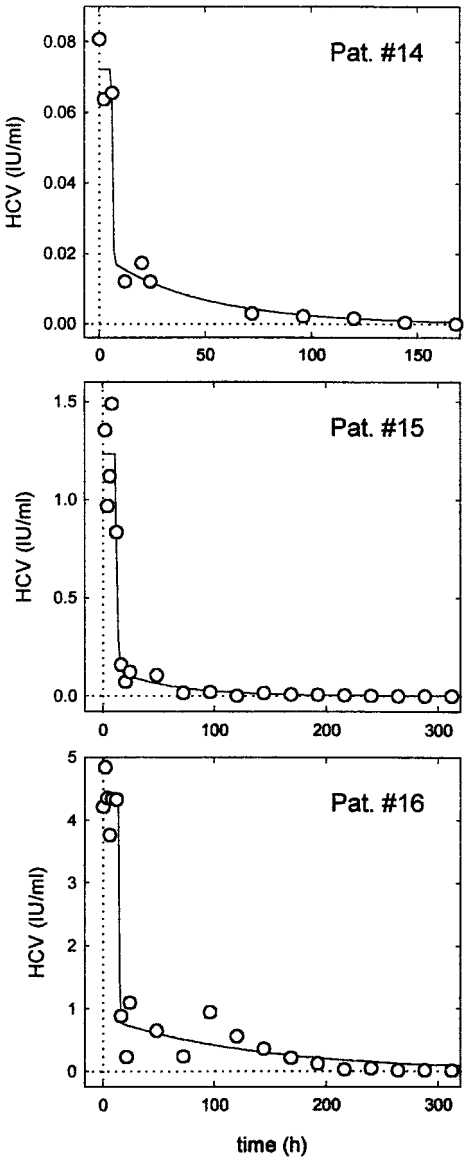

Fig 3. Changes in serum HCV RNA levels during treatment with the triple combination of interferon, ribavirin, and amantadine.

at the end of three months. Serum HCV RNA was still present in all eight monotherapy patients, compared to two of five (40%) on dual therapy and only one of three (33 percent) on triple therapy. In one patient on triple therapy in whom HCV RNA reappeared in serum, increasing the freuqency of interferon injections from three times to six times a week resulted in loss of HCV RNA from serum. The patient subsequently had a sustained virological response. In a second patient, end treatment HCV RNA was negative in serum, but relapsed one month after treatment was discontinued. The third patient discontinued therapy prematurely at five months.

Cloning, Sequencing, and Phylogenetic Analysis of E2 Gene PCR Products. We analyzed samples of sera from patient 1 with clearly demonstrated HCV RNA rebound. PCR products from five time points were cloned and 10 clones from each time point were sequenced, for a total of 50 sequences. Prior to analyses, plasmid sequences promixal and distal to either end of the insertion site were removed. Sequences containing gaps, deletions, or artifacts due to mispriming were excluded. A total of 15 sequences were discarded, providing 35 analyzable sequences, each 196 nucleotides in length. Double-stranded PCR products inserted into the plasmid in the 3′–5′ position were reversed and the complementary sequence determined using a reverse-complement translator (http://arep.med.harvard.edu/labgc/adnan/projects/Utilities/revcomp.html) as necessary. Data from the independent clones from 0 hr ($N = 8$), 12 hr ($N = 8$), 24 hr ($N = 6$), day 5 ($N = 6$), and day 10 ($N = 7$) were analyzed.

Diversity and Evolutionary distances in E2 HVR1. The deduced amino acid sequences translated and aligned using molecular evolution genetics analysis (MEGA software) (14) are shown, with the published HCV-1 prototype, HCV J1 sequence (Table 2). The table also shows the deduced amino acid sequences 359–422, which include the HVR1 sequences 384–410. The HVR1 sequences contain the amino acids threonine, T 385; glycine, G 406; glycine G 389 and glutamine, Q 409, which have recently been noted to be 100% conserved among HCV subtypes (15).

We estimated the average genetic distance within each time point (group) for both the nucleotide (Table 3) and the deduced amino acid sequences (Table 4). The average genetic distance within both nucleotide and amino acid sequences before treatment (0 hr) was small, suggesting a homogeneous, stable viral population (16, 17, 18). The amino acid variability decreased to complete homogeneity by 12 hr, in line with a marked reduction in viral load. This presumed haplotype-independent culling was followed by a gradual increase back to baseline levels by day 5 and to above baseline by day 10 (Figure 5).

DISCUSSION

An analysis of the rapid changes that take place immediately after the initiation of antiviral therapy has provided valuable information regarding the pathogenesis and treatment of chronic viral infections and has helped to elucidate mechanisms of antiviral drug action (3, 5, 19).

The reduction in serum HCV RNA within less than 8 hrs that follows the initial subcutaneous injection of high-dose interferon (figure 1a) has been previously

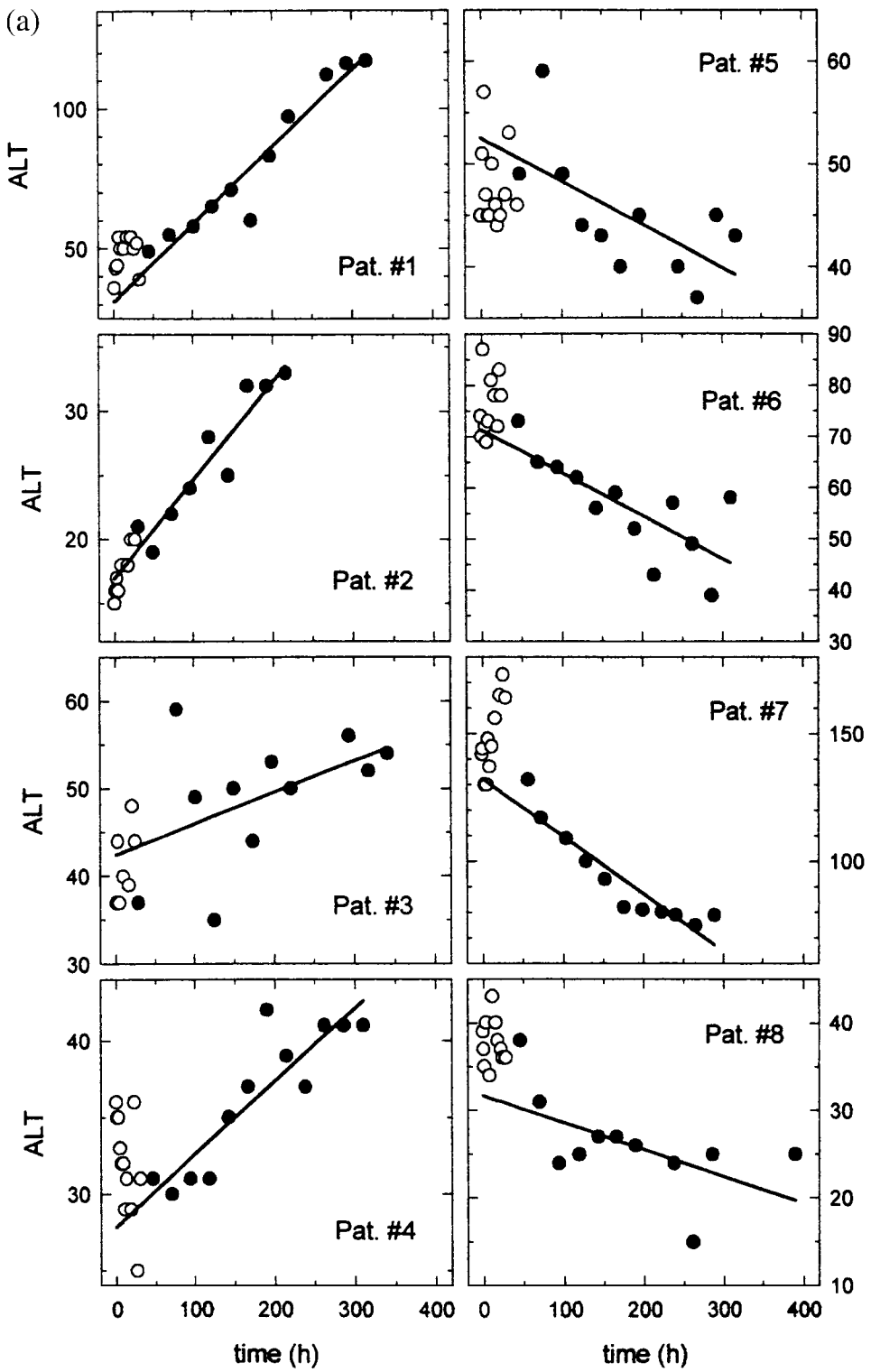

Fig 4. Changes in the ALT levels for (a) patients treated with interferon alone and (b) patients treated by the interferon plus ribavirin combination therapy. The data points highlighted, corresponding to phase 2 (2–14 days) of therapy, were subjected to linear regression analysis to estimate the linear rate of increase or decrease summarized in Table 1 (see column r_{ALT}). Solid lines represent the best linear least squares fit (note the differences in the scale of the ALT levels) which are drawn to indicate the direction of the slope only.

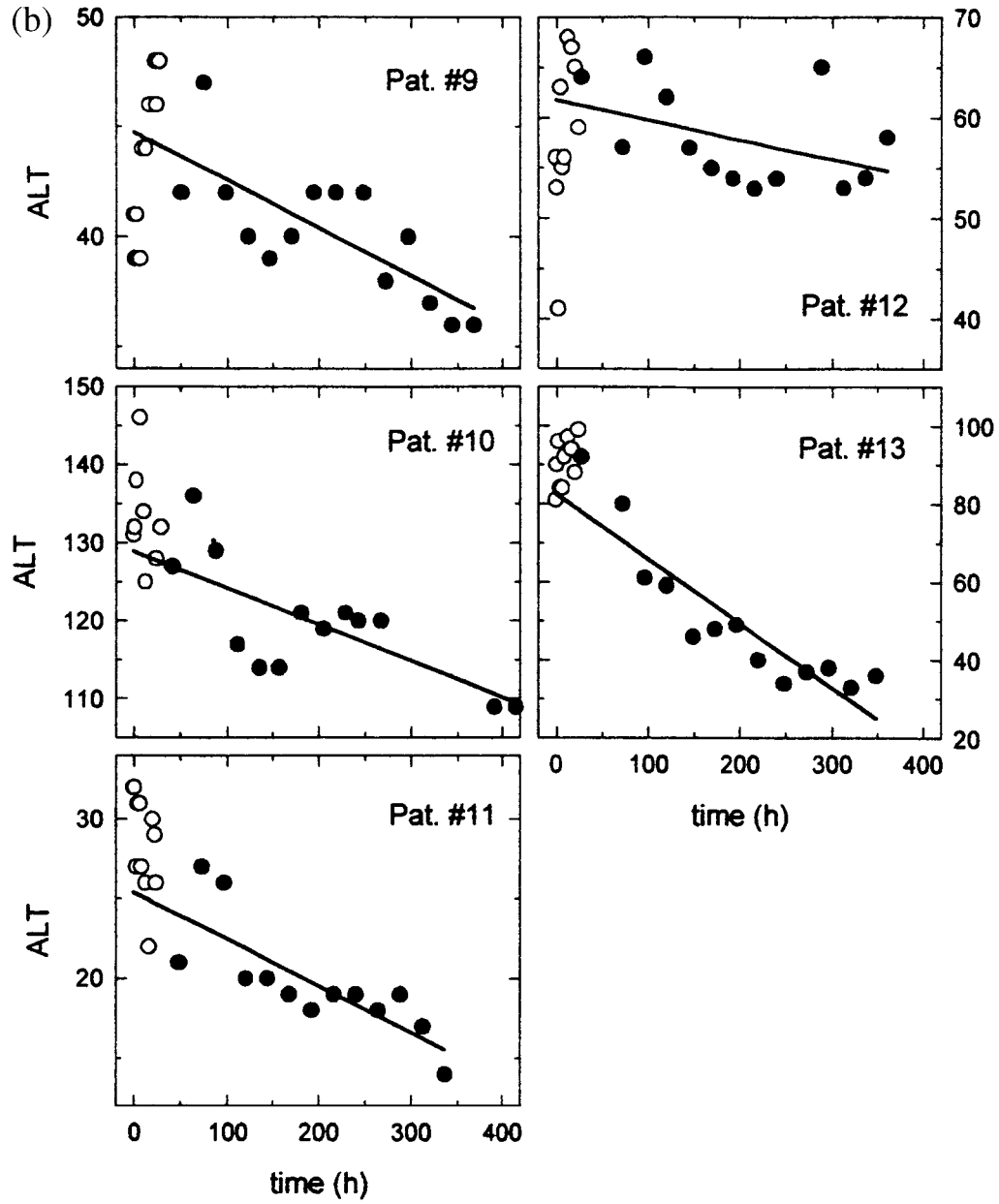

Fig 4. (Continued).

reported by others (5, 8, 10, 20), and is most consistent with direct inhibition of viral replication or release (5). It contrasts sharply with the long delay of several days that preceeds the decline in serum HIV RNA during therapy with HIV protease. In contrast to interferon, HIV protease mainly inhibits de *novo* infection (3, 19).

Serum HCV RNA viral load is maintained by release of virus from HCV-infected cells. The pool of infected cells is sustained by continued *de novo* infection of uninfected target cells. Serum ALT is a surrogate marker that reflects damage to the pool of "old" infected and *de novo* infected cells. Interferon both inhibits viral replication in infected cells and also "interferes" with or partially blocks *de novo* infection of uninfected cells. This explains the rapid initial decline in serum HCV RNA (phase 1) that is followed by a slower phase 2 decline in HCV and ALT as the virus-infected cell pool decreases and is not replenished. This predicted biexponential decay was broadly observed in four of eight monotherapy patients and in all of the

TABLE 2. DEDUCED AMINO ACID SEQUENCES OF INDEPENDENT CLONES AT INDICATED TIMES FROM PATIENT 1*

HCV prototype HCV J1							
#hcv1411-1611	*IAYFSMVGNW*		*AGVDAETHVT*	*GGNAGRTTAG*	*LVGLLTPGAK*	*QNIQLINTNG*	
AA numbering	*359*	*AKVLVVLLLF*	*385†*	*389†*	*406†*	*409†*	*SWHI*
HCV clones pre and post INF							
#LR1-0 hr_(0_hr)	IAYFSMVGNW	AKVLVVLLLF	AGVDAQTYVS	GGSVAQAAFG	LSNLFTPGAR	QNIQLINTNG	SWHI
#LR2-0 hr_(0_hr)	.....	.....	.....	.....	.....	.....	....
#LR3-0 hr_(0_hr)	.....	.....	.....	.....	.T.....	.....	....
#LR5-0 hr_(0_hr)	.....	.....	.....	.....	.....	.....	....
#LR7-0 hr_(0_hr)	.....	.....	.....	.....R....	.....	.....	....
#LR8T3-0 hr_(0_hr)	.....	.....	.....	.....	.....	.....	....
#LR9T3-0 hrgap_(0_hr)	.....-....	.....	.....	.....	.....	.....	....
#LR10T3-0 hr_(0_hr)	.....	.....	.....	.....	.....	.....	....
#LR13-12 hr_(12_hr)	.....	.....	.....	.....	.....	.....	....
#LR14-12 hr_(12_hr)	.....	.....	.....	.....	.....	.....	....
#LR15-12 hr_(12_hr)	.....	.....	.....	.....	.....	.....	....
#LR16T3-12 hr_(12_hr)	.....	.....	.....	.....	.....	.....	....
#LR17T3-12 hr_(12_hr)	.....	.....	.....	.....	.....	.....	....
#LR18T3-12 hr_(12_hr)	.....	.....	.....	.....	.....	.....	....
#LR19T3-12 hr_(12_hr)	.....	.....	.....	.....	.....	.....	....
#LR20T3-12 hr_(12_hr)	.....-....	.....	.....	.....	.....	.....	....
#LR21-24 hr_(24_hr)	.....	.....	.....	.....	.T.....	.....	....
#LR22-24 hrgap_(24_hr)	.....-....	.....	.....	.....	.....	.....	....
#LR23-24 hr_(24_hr)	.....	.....	.....	.....	.T.....	.....	....
#LR24-24 hr_(24_hr)	.....	.....	.....	.....	.....	.....	....
#LR26T3-24 hr_(24_hr)	.....	.....	.....	.....	.....	.....	....
#LR28T3-24 hrgap_(24_hr)	.....-....	.....	.....	.....	.T.....	.....	....
#LBLR75–day5mod_(day_5)	.....-..	.....	.....	.....	.....	.....	....
#LR74–day5_(day_5)	.....	.....	.....	.....	.T.....	.....	....
#LR75b–day5_(day_5)	.....	.....	.....	.....	.....	.....	....
#LR78–day5_(day_5)	.....	.....	.....	.....	.....	.....	....
#LR79–day5_(day_5)	.....	.....	.....	.....	.....G	.....	....
#LR80-day5_(day_5)	.....	.....	.....	.....	.....	.....	...?
#LR81-day10_(day_10)	.....	.....S	.....	.....	.....	.....	....
#LR84–day10_(day_10)	.....	.....	.....	.....	.....	.....	....
#LR85-day10_(day_10)	.....	.....	.....E....	.....	.....	.....	....
#LR86-day10_(day_10)	.....	.....	.....	.....	.....	.....	....
#LR87-day10_(day_10)	.....	.....	.....	.....R....	.....	.....	....
#LR89–day10_(day_10)	.....	.....	.....	.....	.....	.....	....
#LR90-day10_(day_10)	.....	.....	.....	.....R....	.....	.....	....

*The corresponding sequences for the prototype HCVJ1 are shown for comparison. The HVR1 sequences are aa 384–410.
†HVR1 conserved amino acids across subtypes (15).

patients on combination treatment. However, in half of our patients on interferon monotherapy, phase 2 was replaced by a sustained increase or rebound in serum HCV RNA, back towards pretreatment levels. This rebound (Figure 2a) has been noted in several published studies on HCV dynamics using daily high-dose interferon monotherapy (5, 8, 10, 20), and its potential for attenuating the slope of the phase 2 decay has been acknowledged (5). It is reminiscent of the rebound in serum HIV RNA during nevirapine monotherapy in HIV disease (3, 19). It is associated with the emergence of a nevirapine-resistant mutation at codon 181 (3, 19) and is suppressed or delayed by combination anti-HIV therapy. (21). We have hypothesized that the early rebound in serum HCV RNA during high-dose interferon monotherapy may similarly be due to early emergence of interferon escape HCV variants, which infect *de novo*, and replicate in target hepatocytes. The data presented here, demonstrating for the first time

TABLE 3. NUCLEOTIDE DIVERSITY OF HVR1 SEQUENCES AT INDICATED TIMES FROM PATIENT 1 ON INTERFERON MONOTHERAPY: EVOLUTIONARY DISTANCES DETERMINED BY KIMURA-2 DISTANCE

	0 hr	*12 hr*	*24 hr*	*5 days*	*10 days*
Distance (within group), mean ± SE	0.008 ± 0.003	0.001 ± 0.001	0.008 ± 0.004	0.007 ± 0.004	0.014 ± 0.005

TABLE 4. AMINO ACID DIVERSITY OF HVR1 SEQUENCES AT INDICATED TIMES FROM PATIENT 1 ON INTERFERON MONOTHERAPY: EVOLUTIONARY DISTANCES DETERMINED BY POISSON DISTRIBUTION

	0 hr	*12 hr*	*24 hr*	*5 days*	*10 days*
Distance (within group), mean ± SE	0.008 ± 0.005	0.00 ± 0.00	0.01 ± 0.009	0.011 ± 0.007	0.017 ± 0.01

that the HCV rebound is associated with a simultaneous and parallel increase in serum ALT (Figure 4a), support this hypothesis.

The addition of ribavirin and amantadine to interferon significantly increased the mean viral clearance rates in both phases 1 and 2. These data confirm a previous report (20). An almost 10-fold increase in phase 2 clearance was observed with the addition of both ribavirin and amantadine to interferon in the present study. These highly significant effects of ribavirin and amantadine on early viral clearance during phase 2 are most consistent with inhibition of *de novo* infection.

Amantadine directly blocks viral entry through the cell membrane (22). Ribavirin (1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide), a nucleoside analog, has inhibitory effects on HCV NS5B RNA dependent RNA polymerase *in vitro* (23). Mild antiviral efficacy could inhibit establishment of *de novo* replication with little effect on viral load and is consistent with the well-described effect of ribavirin monotherapy in clinical practice (24).

Supportive data for an stimultatory effect of ribavirin on the immune system exist (25–27) and some have suggested this as a mechanism for increased phase 2 clearance. However, the rapid effect of ribavirin on early viral clearance more likely indicates antiviral rather than immune stimulatory properties.

Daily HCV production estimated in the current study for interferon monotherapy is similar to that reported by others (5, 7, 9, 27). The more complete inhibition of viral replication provides a more accurate estimate of infected cell death rates. The infected cell death rate in the current study, 2.1–20 days, is similar to the range of 1.5–10.3 days reported by others for combination treatment (8) and is shorter and less variable that for interferon monotherapy (5). The rapid turnover of infected hepatocytes is supported by studies from our group and from others that demonstrate a high percentage of dividing hepatocytes in liver biopsy tissue from patients with chronic HCV liver disease (28–31).

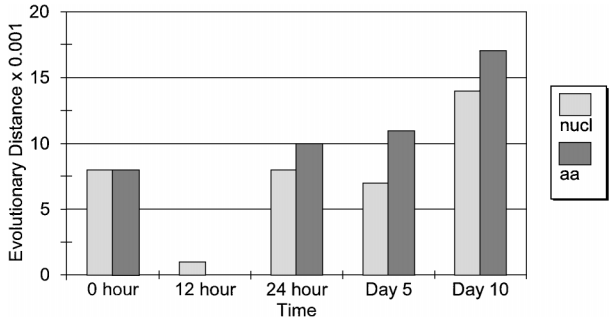

Fig 5. Graphic representation of data in Tables 3 and 4 showing changes in the nucleotide and amino acid diversity of HVR1 sequences during high dose interferon monotherapy in patient 1.

Several studies have shown a low rate of sustained response to interferon therapy in African-American (AA) patients (32–35). The treatment efficacy in the two HCV genotype 1 AA subjects given interferon monotherapy in the present study was significantly lower that in the non AA subjects. Layden et al (36), in a recent reanalysis of their data, observed significantly reduced interferon efficacy in 25 AA subjects compared to a matched group of Caucasians. These preliminary results could begin to shed light on potentially important mechanisms underlying the low response rate in AA population to interferon treatment.

The transient increase or "hump" in HCV RNA, peaking around 120 hr after initiation of combination treatment with ribavirin (Figure 2b, patients 12 and 13) has been reported previously (20, 37). The full significance of this transient increase is unclear. The hump is significantly attenuated by the addition of amantadine (Figure 3) and suggests more complete inhibition of *de novo* inhibition by triple therapy.

At the end of three months of conventional therapy, none of the patients on monotherapy, two of five patients (40%) on dual therapy and two of three (67%) on triple therapy had cleared HCV RNA from serum. The correlation between early viral clearance and the final outcome of therapy is interesting. However, the design of the present study and the small numbers do not allow any strong conclusions to be drawn regarding HCV dynamic parameters and the long-term clinical response or outcome.

Validation of our hypothetical model would depend on a demonstration that rapid evolutionary changes in the HCV quasispecies population can occur immediately after initiation of interferon therapy. We chose to study this by sequencing the N-terminus of the second envelope glycoprotein (E2) region, the HVR1, where maximal nucleotide and amino acid replacements take place. Although HVR1 is unlikely to be a target site for early interferon resistance

selection, linkage or development in tandem with HVR1 or other alleles with regions within the HCV genome that determine interferon resistance may allow these sequences to hitchhike along and mirror the interferon resistance determining regions (38). The HCV variants isolated from the pretreatment serum sample from our patient (patient 1) showed relatively low variability, indicating a relatively fit starting virus replicating in a stable environment. The reduction to complete homogeneity within less than 12 hr (Table 2) probably reflects the sharp reduction in viral load. A gradual increase in amino acid and nucleotide diversity then followed (Figure 5) and is consistent with possible evolution of new variants.

The complex interactions between virus and host, early in the course of the acute viral infection, including hepatitis C virus infection (39) determines in large part resolution or persistence of the chronic infection. The results reported here for the first time demonstrate that significant evolutionary changes take place within hours of the first does of interferon. This study design should prove to be a useful model for sensitively and correctly identifying interferon resistance determining regions in the HCV genome.

In summary, the early effect of ribavirin and amantadine on serum HCV RNA clearance after interferon is consistent with our hypothesis that these drugs significantly attenuate residual HCV during interferon monotherapy, presumably by reducing *de novo* infection. Sequencing data provide supportive evidence that early interferon escape HCV variants could explain the rebound and lower viral clearnce rates observed during interferon monotherapy. Although the number of patients included in these studies is small, these data provide a preliminary explanation for a possible mode of action of ribavirin and amantadine.

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