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Human Serum Facilitates Hepatitis C Virus Infection, and Neutralizing Responses Inversely Correlate with Viral Replication Kinetics at the Acute Phase of Hepatitis C Virus Infection

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The factors leading to spontaneous clearance of hepatitis C virus (HCV) or to viral persistence are elusive. Understanding virus-host interactions that enable acute HCV clearance is key to the development of more effective therapeutic and prophylactic strategies. Here, using a sensitive neutralization assay based on infectious HCV pseudoparticles (HCVpp), we have studied the kinetics of humoral responses in a cohort of acute-phase patients infected during a single nosocomial outbreak in a hemodialysis center. The 17 patients were monitored for the spontaneous outcome of HCV infection for 6 months before a treatment decision was made. Blood samples were taken frequently (15 ± 4 per patient). Phylogenetic analysis of the predominant virus(es) revealed infection by only one of two genotype 1b strains. While all patients seroconverted, their sera induced two opposing effects in HCVpp infection assays: inhibition and facilitation. Furthermore, the ability of sera to facilitate or inhibit infection correlated with the presence of either infecting HCV strain and divided the patients into two groups. In group 1, the progressive emergence of a relatively strong neutralizing response correlated with a fluctuating decrease in high initial viremia, leading to control of viral replication. Patients in group 2 failed to reduce viremia within the acute phase, and no neutralizing responses were detected despite seroconversion. Strikingly, sera of group 2, as well as naïve sera, facilitated infection by HCVpp displaying HCV glycoproteins from different genotypes and strains, including those retrieved from patients. These results provide new insights into the mechanisms of viral persistence and immune control of viremia.

Hepatitis C virus (HCV) infection causes acute hepatitis after 4 to 12 weeks of incubation. Acute hepatitis is characterized by elevated alanine aminotransferase (ALT) levels, with generally no or only mild symptoms. Among infected individuals, only 20% clear infection spontaneously, whereas ~80% progress to chronic infection. Chronic hepatitis may lead after 10 to 30 years to severe, life-threatening complications, such as cirrhosis and hepatocellular carcinoma. With an estimated 170 million people infected, i.e., nearly 3% of the world population, and an incidence of ~3 to 4 million new infections per year, HCV is presently a leading cause of chronic liver disease and poses a major public health problem. In the United States, HCV constitutes the most common chronic blood-borne infectious disease and is the principal indication for liver transplantation and the 10th leading cause of deaths among adults. The only approved therapy for chronic hepatitis C is the combination of alpha interferon, used in a pegylated form, and ribavirin. This treatment cures infection in a significant proportion of patients, but its efficacy against HCV genotype 1, the most frequent HCV genotype in industrialized countries, remains limited, and it can cause significant side effects (18, 23, 36). HCV is a highly variable virus that comprises six main genotypes and >100 subtypes and evolves into viral quasispecies in infected individuals (42). This renders the design and development of specific HCV inhibitors difficult and explains the fact that no efficient vaccine has been developed.

Better knowledge of the viral and host factors that determine HCV clearance or persistence at the acute stage of infection is needed in order to improve antiviral therapy and develop efficient vaccines. Studies focusing on innate and cellular immune responses have shown that a sufficiently large HCV inoculum is able to evade, subvert, or circumvent the defenses of the host. Thereafter, spontaneous HCV clearance is associated with a strong early cellular immune response to multiple HCV epitopes (10, 11, 57), and both $CD4^+$ and $CD8^+$ responses are maintained for several years after viral clearance (55). Conversely, a loss of the $CD4^+$ response can result in recurrence of HCV infection (20), whereas nonsustained and/or dysfunctional HCV-specific $CD4^+$ and $CD8^+$ responses have been associated with HCV persistence (22, 34). In contrast, the role of humoral immunity at the acute stage of HCV infection has been suggested in several studies but remains poorly characterized (9, 27, 48, 57). Detection of neutralizing antibodies in patients' blood has been difficult, owing to the lack of an efficient and reliable cell culture system for HCV. Neutralizing antibodies have, however, been identified by their ability to prevent both HCV replication in a lymphoid cell line and HCV infection in experimentally inoculated chimpanzees

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(14, 16, 51, 52) or, alternatively, by their capacity to inhibit binding of E2 to CD81 (41), an HCV receptor candidate. It is now widely accepted that neutralizing anti-HCV antibodies are present in the blood of chronically infected patients. They have been reported to emerge during the course of acute HCV infection (52) both in human patients and in experimentally infected chimpanzees (14, 16). Neutralizing responses remained difficult to measure precisely. Using a novel in vitro neutralization assay system based on infectious retroviral pseudoparticles bearing HCV envelope glycoproteins (HCVpp) (3, 26), we and others were able to confirm that HCV-infected patients' blood neutralizes in vitro infection. With this quantitative assay, high-titer neutralizing antibody levels were detected in plasmas from chronically infected chimpanzees and humans (2, 3, 26, 33, 35).

Here, we characterize the kinetics of humoral immune responses in a cohort of acutely infected hemodialysis patients infected by a single viral strain during a nosocomial outbreak in a hemodialysis center with various clinical and virological outcomes. This approach allowed us to demonstrate for the first time (i) the presence of neutralizing antibodies at the acute phase of HCV infection, (ii) the inverse correlation of the emergence of neutralizing responses with HCV RNA kinetics, and (iii) the existence of human blood components that facilitate HCV infection.

MATERIALS AND METHODS

Materials. 17 hemodialysis patients (6 males and 11 females; mean age, 63.7 years; range, 37 to 77 years) from the Department of Renal Disease at Papageorgiou General Hospital, Thessaloniki, Greece, were found to have ALT elevations on routine screening and were subsequently found to be HCV RNA positive in the context of an HCV outbreak in the hemodialysis ward. All of them were included in a cohort study aimed at monitoring the spontaneous outcome of their acute infection over 6 months before deciding to eventually start antiviral therapy. This study was approved by the Institution Review Board and Ethics Committee of the Papageorgiou General Hospital, and the patients gave their informed consent to be included in the study. Blood samples were taken frequently during the study period (on average, 15 ± 4 samples per patient; range, 8 to 22 samples) and frozen at −80°C. These serum samples constituted the materials for this study. Blood sampling was systematically performed just before hemodialysis in order to avoid the effects of hemodialysis on the various parameters studied. All blood samples were coded, and all information pertaining to this cohort of patients was maintained on a computer database with password access restricted to the principal investigator.

Assessment of ALT levels, anti-HCV antibodies, and HCV RNA kinetics. ALT levels were determined prospectively. Seroconversion was characterized over the course of infection by means of a line immunoassay detecting and semiquantifying antibodies directed to eight HCV antigens located in the core, E1, E2, NS3, NS4, and NS5 regions (INNO-LIA HCV IV prototype assay; Innogenetics, Ghent, Belgium) according to the manufacturer's instructions. Briefly, diluted samples were incubated with the LIA test strip, which had been coated with HCV antigens. Specific HCV antigen-antibody complexes were detected with a labeled anti-human immunoglobulin (Ig). The enzyme substrate produced a color, the intensity of which was proportional to the amount of anti-HCV antibody captured from the sample. Finally, HCV RNA kinetics were characterized over the course of acute infection by measuring HCV RNA levels in all patients' samples by means of a third-generation branched-DNA-based assay (Versant HCV RNA 3.0 Assay; Bayer Diagnostics, Tarrytown, N.J.).

Sequence and phylogenetic analyses. In order to determine the HCV genotype and whether the patients were all infected with the same HCV strain or different strains circulating simultaneously during the hepatitis C outbreak, a fragment of the NS5B encoding region and hypervariable region 1 (HVR1) of the E2 envelope glycoprotein-encoding region were directly sequenced in the first available sample from all patients. In addition, the full-length E1-E2 region was sequenced in one representative of all the different infecting viral strains, in order to use these sequences to prepare virus-specific HCV pseudoparticles. For this, RNA was extracted from 200 μl of serum using a High Pure Viral RNA kit (Roche Applied Science, Indianapolis, Ind.). The extracted RNA was reverse transcribed for 1 h at 50°C using Superscript III reverse transcriptase (Invitrogen, Carlsbad, Calif.). All of the PCRs were carried out with the Advantage 2 Polymerase Mix (Clontech, Palo Alto, Calif.). PCR amplifications of the E1-E2 region were achieved with upstream (5′GGTCGCGYAATYTGGGTAAGGT3′) and downstream (5′AGCYTGCCYTTRATGTACCAG3′) primers. After denaturation for 1 min at 95°C, PCR comprised 35 cycles (95°C, 30 s; 52°C, 30 s; 68°C, 2 min). HVR1 PCR was performed by using upstream (5′GCTTGGGATATGATGAT GAACTGGTC3′) and downstream (5′GTCCTATTGATGGTGCCARCT3′) primers and comprised 35 cycles (95°C, 30 s; 55°C, 30 s; 68°C, 30 s). Touchdown PCR was carried out as described previously (38). All of the PCR products were purified using Montage PCR filters (Millipore, Billerica, Mass.), and HVR1 and NS5B amplicons were directly sequenced. Purified E1-E2 PCR products were cloned into the pCRII vector (TA cloning kit; Invitrogen). The cloned DNA was reamplified by using universal M13 primers for sequencing. Nucleotide or amino acid sequences were aligned using the Clustal W program, version 1.8 (58). PHYLIP (Phylogenetic Inference Package) software, version 3.573 (31), was used to construct phylogenetic trees by means of the neighbor-joining method with a sequence matrix determined by the Kimura two-parameter method. Bootstrap support was determined by 1,000 resamplings of the sequences. The following sequences from prototype HCV strains of different genotypes were also included in the analyses: H77 (AF009606), HCV-BK (M58335), Con1 (AJ238799), CG (AF333324), HC-G9 (D14853), HC-J6 (D00944), NZL1 (D17763), ED43 (Y11604), EUH1480 (Y13184), and EUHK2 (Y12083).

Production of HCVpp and in vitro infection experiments. HCVpp were generated as described previously (3, 26). Briefly, 293T cells were transfected with three expression vectors encoding the HCV envelope glycoproteins, core and packaging components from murine leukemia virus, and a murine leukemia virus retroviral transfer vector harboring a marker gene encoding the green fluorescent protein (GFP). Expression plasmids encoding both HCV E1 and E2 glycoproteins of genotype 1b strain CG1b (AF333324) (59), BK (M58335) (56), or UKN1B.12.6 (AY734975) (33) or virus recovered from patients of this cohort (HCV strains A and B), or of genotype 1a strain H77 (28, 39) (AF009606) were used. Supernatants containing HCVpp were harvested 24 h after transfection, filtered through 0.45-μm-pore-size membranes, and used to infect Huh-7 cells (8×10^4 cells/well in 12-well plates). Serum samples (human samples or, as a control, fetal calf serum [FCS]) were diluted 1/50 and tested for neutralization of 10^4 infectious unit (i.u.) of HCVpp by preincubating the mixture at room temperature for 30 min before adding it to the target cells. After 3 h, the supernatants were removed and the cells were incubated in regular medium for 96 h at 37°C, at which time GFP expression was determined by fluorescence-activated cell sorter analysis. The residual infectivity of HCVpp incubated with human serum was compared with the HCVpp infectivity upon incubation with a medium containing FCS. Input pseudoparticles infected 15 to 20% of the cells in the absence of human serum. The infectivity of HCVpp exposed to culture medium without human serum was standardized to 100% for comparison with the patients' samples. The percentages of neutralization were calculated as the means of at least three independent experiments. The positive control sample was from a French patient (Vu) with chronic HCV genotype 1b infection. The neutralization titers were determined by serial twofold dilutions of sera followed by incubation with HCVpp of genotype CG1b, as previously described (2). Negative control neutralization experiments were also performed in parallel with an irrelevant target by using pseudoparticles bearing glycoproteins derived from the feline endogenous retrovirus RD114 as described previously (3) (antibodies to this virus are not found in human sera [2]). Additional control experiments were performed with pseudoparticles bearing glycoproteins from other enveloped viruses, such as the hemagglutinin of fowl plague virus and the G protein of vesicular stomatitis virus (VSV-G) (50).

Nucleotide sequence accession numbers. The sequences of the predominant viruses retrieved from some patients have been submitted to GenBank under accession numbers AJ849943 through AJ849974.

RESULTS

A unique cohort of acute-phase patients infected with a dual HCV source. Our cohort consisted of 17 hemodialysis patients with HCV RNA-positive acute HCV infection who had all been infected during a nosocomial outbreak that occurred in the hemodialysis ward of the Papageorgiou General Hospital (Thessaloniki, Greece) in mid-2002. The outbreak covered a

maximum period of 6 weeks, and the routes of infection have not been identified. One patient was found to have seroconverted to HCV after an ALT elevation was discovered on systematic screening. This led to testing of the other patients from the hemodialysis ward. Overall, 17 patients (6 males and 11 females; mean age, 63.7 years; range, 37 to 77 years) were found to have been infected and to be HCV RNA positive. The date of infection, and hence the time between infection and entry into the study, could not be precisely determined for these patients, who were undergoing several hemodialysis sessions per week. They were all followed for 6 months without treatment before a therapeutic decision was made, with the exception of four patients (Pt-4, Pt-9, Pt-11, and Pt-15) who underwent treatment before the end of the 6-month follow-up for clinical reasons (see below). Acute HCV infection was confirmed in all patients by characterizing seroconversion profiles with a line immunoassay that semiquantitatively detects antibodies directed to eight HCV antigens, including the core, E1, E2, NS3, NS4, and NS5 proteins. As shown in Fig. 1, except for patients Pt-13 and Pt-16, who exhibited unchanged and incomplete antibody profiles over the full study period, all other patients developed progressively increasing titers of anti-HCV antibodies typical for a seroconversion period (Fig. 1), confirming that they were at the acute stage of HCV infection. As also shown in Fig. 1, various ALT elevation profiles were observed, including patients with maximal ALT elevation in the first available samples (e.g., Pt-5, Pt-6, and Pt-13) and patients exhibiting an ALT peak later on during follow-up (e.g., Pt-1, Pt-3, and Pt-9).

In order to determine whether the study patients had been infected by a single HCV source during the outbreak, we directly sequenced a 329-bp fragment in the NS5B region in the first available serum sample from each patient. These sequences were compared among the different patients and with reference HCV sequences (data not shown). Phylogenetic analyses showed that the patients were in fact infected with either of two distinct HCV genotype 1b strains (HCV strain A, nine patients; HCV strain B, seven patients; one patient [Pt-14] could not be genotyped). Analysis of the full sequences of the E1-E2 glycoproteins from viruses representative of both strains, from patients Pt-3 and Pt-8 (infected by strain A) and from patients Pt-5 and Pt-12 (infected with strain B), were consistent with those of the NS5B region and confirmed the classification of the infecting viruses into two distinct strains within the genotype 1b cluster (Fig. 2A). Thus, although the source patient(s) could not be identified, two distinct HCV-1b strains circulated within the hemodialysis ward during the outbreak. Figure 2B shows the HVR1 amino acid sequences (based on direct sequence analysis) retrieved from the first available sample from the patients infected with HCV strains A and B. While almost no differences were detected in the HVR1 sequence of the E2 envelope glycoproteins among the patients infected with strain A, suggesting a lack of selection pressure and slow genetic evolution, some differences were detected in the HVR1 regions of patients infected with strain B (Fig. 2B), which could be due to a limited evolution of this virus into quasispecies. Overall, this cohort of untreated hemodialysis patients with acute hepatitis C was iatrogenically infected with a dual HCV source and represented a unique opportunity to study the relationship between neutralizing responses and viral kinetics in the context of seroconversion.

Human sera can induce two opposite effects on HCV infectivity: inhibition and facilitation. To monitor the effect of human sera on HCV infectivity, we used an in vitro HCV infection assay that we and others recently described (3, 12, 26). This assay consists of measuring the effect of human serum on the in vitro infectivity of HCVpp that are generated by coating retroviral core particles harboring a marker gene (GFP) with unmodified HCV E1-E2 glycoproteins. Analysis of expression of the marker gene in infected cells readily allows the determination of the effects of sera incubated with the HCVpp before infection (2). At least three independent experiments using different virion production batches were performed for each point of the kinetics shown in Fig. 1, and the standard variations determined to assess for interassay reproducibility did not exceed 30% (mean of the standard variations, 8%), in agreement with our previous reports (2, 62). The results were expressed as percentages of residual infectivity upon HCVpp-serum incubation on Huh-7 cells (Fig. 3 and 4) or, to ease the reading of neutralization curves overlaid with the HCV load and ALT kinetics, as percentages of neutralization (Fig. 1). Note that since the sera of patients before infection were not available, it was not possible to perform the neutralization experiments with patients' sera by comparison with normal sera. The experiments were therefore performed by comparison with FCS. Finally, to emulate the sequences of the predominant strains found in the patients' sera with those of the glycoproteins displayed on the HCVpp (Fig. 2A), we used the E1-E2 sequences from a genotype 1b infectious clone of strain CG1b (59).

As shown in Fig. 1A, sera of some patients at the acute stage of HCV infection exhibited varying levels of inhibition of HCVpp infectivity, which we interpreted as neutralizing serum responses. Depletion of IgG from patients' sera dramatically reduced the inhibition of HCVpp infectivity (Fig. 4), indicating that neutralization was mediated by antibodies. Relatively strong neutralizing responses, with titers of up to 400 and 100, as determined by 50 and 90% inhibiting doses, respectively, could be measured in some of these sera. The individual kinetics of the patients are described in detail below. To test the extent of the neutralizing response in acute-phase patients, we investigated the ability of the patients' sera to cross-neutralize HCVpp derived from alternative HCV strains and/or genotypes. The infectivity of HCVpp generated with the E1-E2 glycoproteins from HCV strain BK (56), which has the closest homology to strain CG1b (Fig. 2A), was inhibited at similar levels, and the kinetic patterns were compared to those found for HCVpp-CG1b, as shown in Fig. 3 using sera from patients Pt-1 and Pt-6, infected with HCV strain A (note that for reasons explained below, the left *y* axis of Fig. 3 indicates the percent infectivity rather than percent neutralization, as in Fig. 1). Likewise, efficient neutralization of HCVpp generated with the E1-E2 glycoproteins from HCV strains A and B could be demonstrated (Fig. 3). Evidence for cross-neutralization was also found with HCVpp-UKN1B.12.6, harboring E1-E2 glycoproteins from a different genotype 1b strain (Fig. 2A). However, neutralization was much less marked than for the former HCVpp-1b and was limited to a few sera, for example, that of patient Pt-6 at week zero (Fig. 3). Some sera from patients

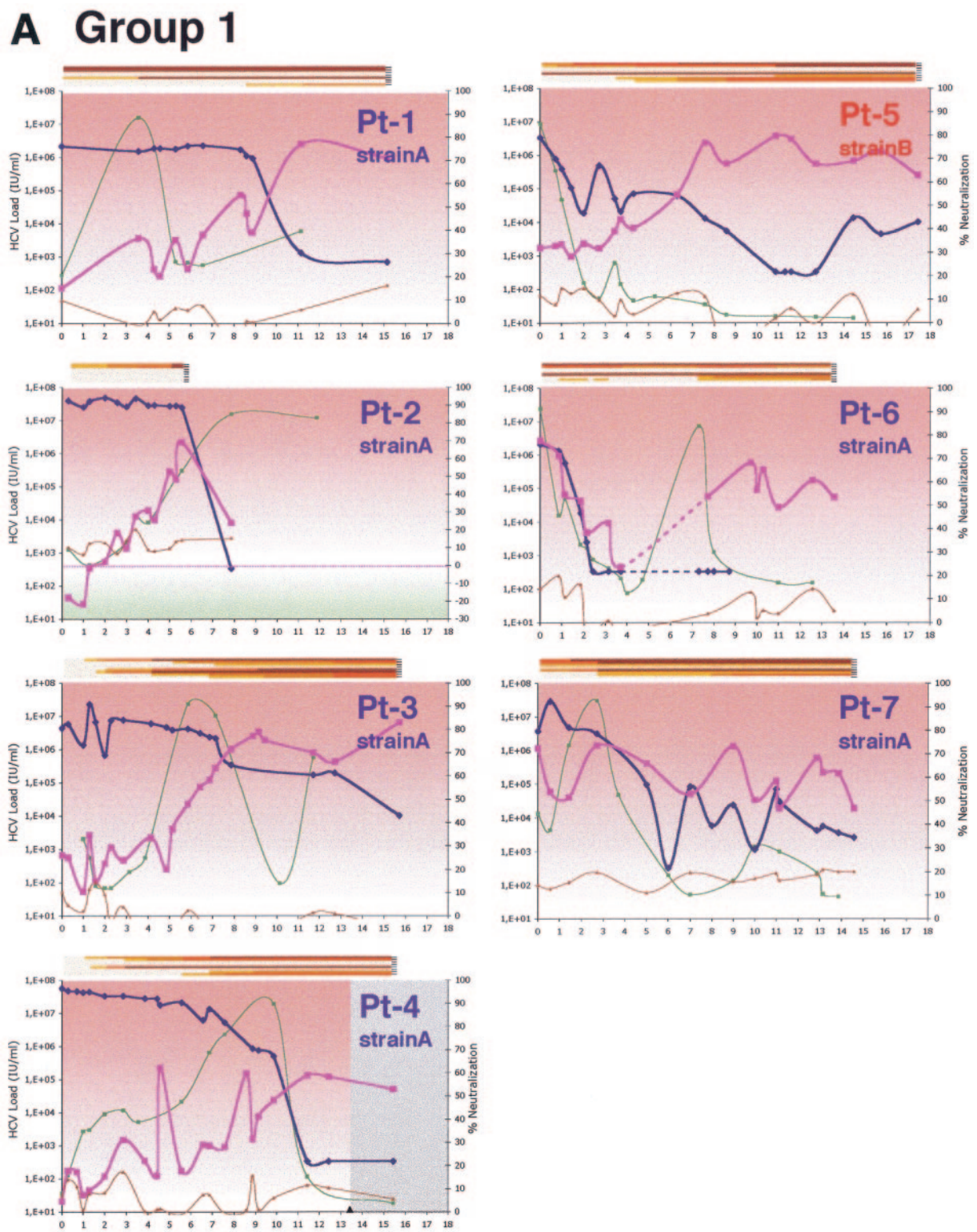
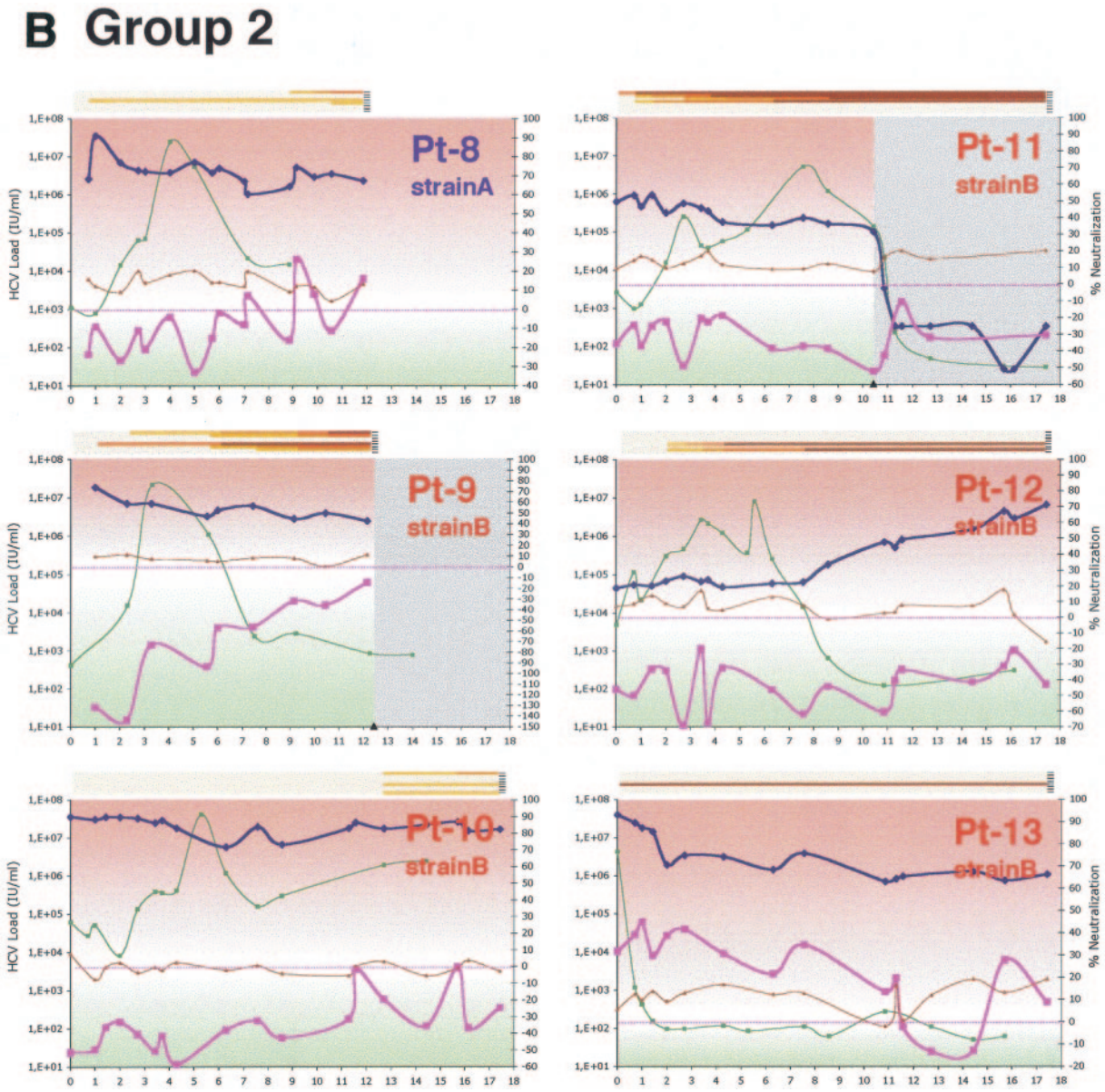


FIG. 1. ALT levels, seroconversion patterns, and kinetics of HCV RNA and of neutralizing and facilitating response levels in our cohort of acutely HCV-infected hemodialysis patients. Individual ALT kinetics (green curves; arbitrary units) and HCV RNA kinetics (blue curves) were measured approximately on a weekly basis following inclusion of the patients in the cohort. The individual patients' kinetics are shown. The coded names for patients infected with virus strain A and strain B (Fig. 2) are highlighted in blue and red, respectively. Patients from group 1 (Fig. 1A) exhibited significant HCV RNA fluctuations that ultimately led to control of viral replication. Patients from group 2 (Fig. 2B) exhibited sustained high replication levels throughout the entire study period. Seroconversion patterns were characterized with the INNO-LIA HCV IV line immunoassay that detects antibodies against HCV structural and nonstructural proteins. The appearance of and increase in antibody titers are shown as colored lines (color code: yellow, low antibody titer, to brown, high antibody titer) at the top of each patient's diagram for the eight tested



HCV antigens (from top to bottom: core 1, core 2, E1, E2, NS3, NS4A, NS4B, and NS5). The effect of each serum sample on the infectivity of HCV genotype 1b pseudoparticles (HCVpp, strain CG1b) was analyzed by incubating identical ratios of viral particles (10^4 i.u.) and sera (1/50 dilution) for 30 min at room temperature before infection of Huh-7 target cells. The results (pink curves) are expressed as the mean percentages of inhibition of the average infectious titers relative to incubation with medium devoid of human serum. The results were derived from at least three independent experiments using different virion production batches, and the standard deviations (not shown for sake of clarity) did not exceed 30% of the mean values. Since some sera facilitated infection, the resulting infectivity was higher than that of HCVpp incubated with human serum-free medium and consequently raised negative values when expressed as percentages of inhibition. The baseline that separated neutralization (red areas; positive values) and facilitation (green areas; negative values) is shown as dotted lines. The specificity of either phenomenon was addressed by using control pseudoparticles generated with the irrelevant target RD114 glycoprotein from a feline endogenous virus (RD114pp), for which no antibodies are detected in human sera (3). As expected, nonspecific inhibition or facilitation of the control RD114pp (brown curves) was never detected over a value of ±20%. Patients Pt-4, Pt-9, and Pt-11 were treated with pegylated alpha interferon a, 135 μg weekly, during the follow-up period for various reasons explained in the text (gray areas), resulting in significant HCV RNA load decreases.

infected with strain A were found to contain cross-neutralizing antibodies that also inhibited HCVpp-H77, of genotype 1a, as shown in Fig. 3 for patient Pt-6 at week zero. Overall, such cross-neutralizing antibodies were detected only in the sera that best neutralized genotype 1b HCVpp. In contrast to sera from most patients infected with HCV strain A, sera from most of the strain B-infected patients could not neutralize HCVpp (Fig. 1B), whatever the genotype and/or strain, even when HCVpp displayed autologous, patient-derived E1-E2 glycoproteins, as shown, for example, for sera from patient Pt-11 (Fig. 3). Altogether, these results indicated overall that while acute-phase patients infected with HCV of strain A elicited an effi-

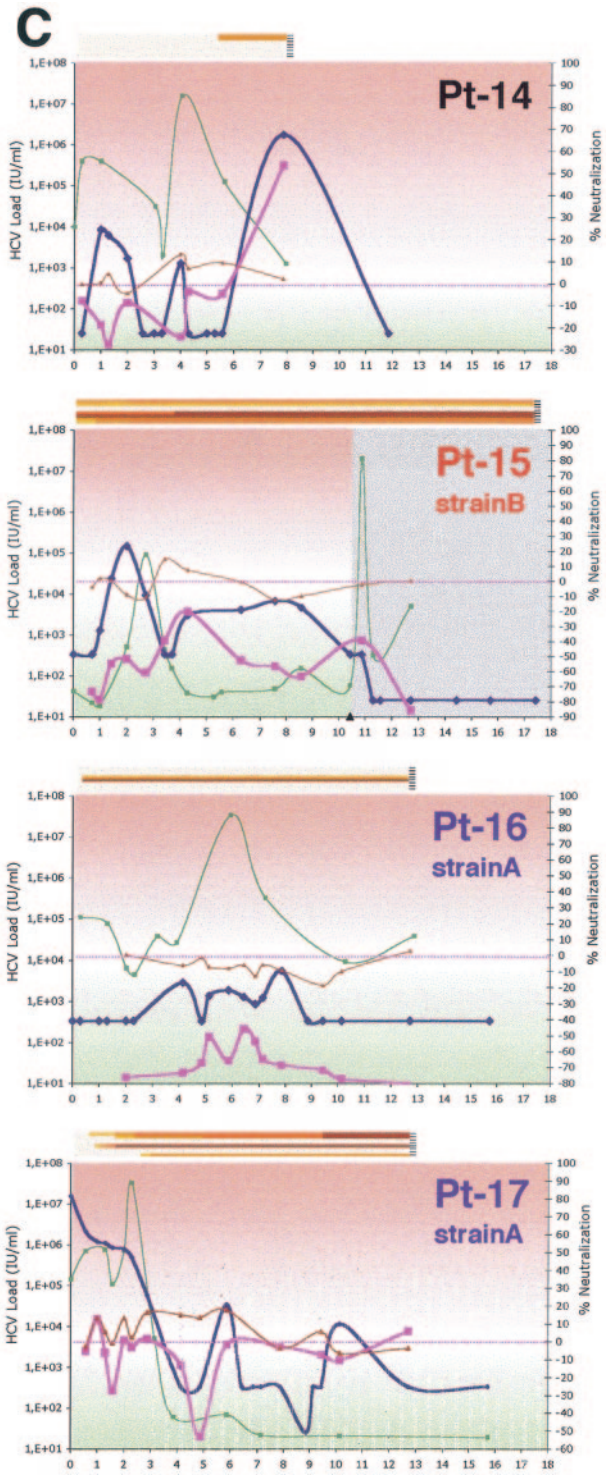


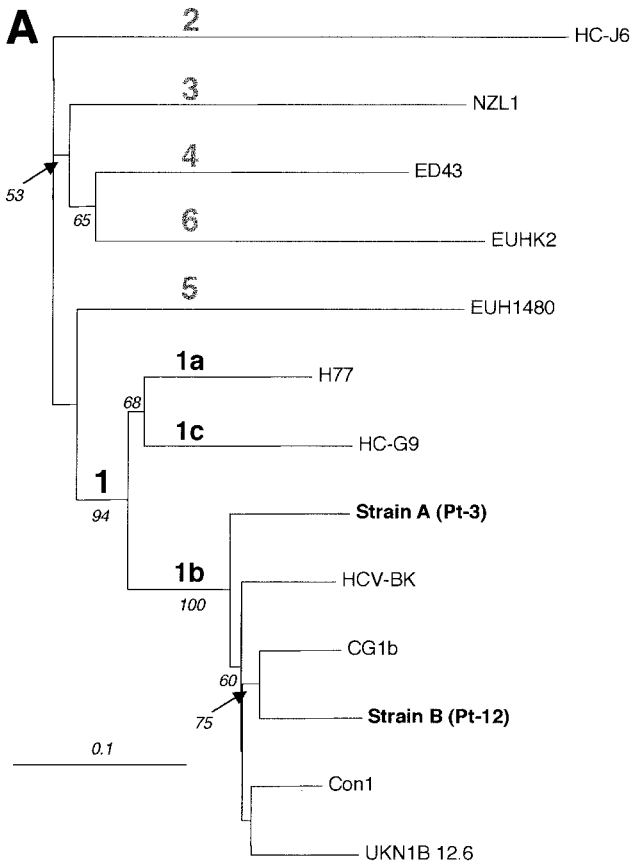
FIG. 1—*Continued*.

cient, though narrowly reactive, neutralizing response, patients infected by the strain B virus did not elicit neutralizing antibodies despite their seroconversion.

Importantly, most of the latter group of sera were found to facilitate infection of HCVpp. This is indicated in Fig. 3 and 4 by the residual HCVpp infectivity upon serum incubation, which was >100%, and consequently, by negative values on the percent neutralization scales of Fig. 1B. Depending on the sera and/or HCV strains, facilitation could reach up to threefold enhancement of infection compared to infection performed in the absence of human serum (Fig. 4). This enhancement was not mediated by the complement, as described for other infectious agents, because heat treatment did not eliminate the effect (data not shown). Furthermore, facilitation of infection was not specific for serum samples of HCV carriers. Indeed, as shown in Fig. 4, sera from uninfected blood donors (PS1 to PS4), which tested negative for anti-HCV antibodies, also facilitated infection to an extent similar to that of HCV patients who did not develop detectable neutralizing responses (Pt-8, Pt-9, and Pt-10). The facilitating effect of the sera was not mediated by human immunoglobulins, as IgG-depleted sera exerted similar or higher enhancement (Fig. 4 and data not shown), and was specific for HCVpp. Indeed, facilitation was not detected with pseudoparticles harboring alternative glycoproteins, such as those derived from the feline endogenous virus RD114, influenza virus (fowl plague virus), or VSV (Fig. 4). Finally, the amplitude of facilitation depended on the HCV genotype and/or strain: HCVpp-1a was more sensitive to facilitation of infection than HCVpp-1b (Fig. 4). Overall, these findings suggested that an unknown component(s) of normal human serum could facilitate HCV infection.

We thus investigated the hypothesis that the effect on HCVpp infectivity observed with a given patient serum is the balance between two opposing forces, i.e., inhibition by neutralizing antibodies and facilitation by an alternative serum component. As shown in Fig. 1A, patient Pt-1's serum capacity to inhibit HCVpp-CG1b infectivity increased with time. Although no neutralizing activity was detected against the latter pseudoparticles in the initial samples (e.g., week zero) (Fig. 3), these early sera were found to facilitate infection by HCVpp-UKN1B.12.6 and HCVpp-H77. Thus, at week zero, HCVpp-serum incubation resulted in 140 to 170% residual infection (or, in other words, 40 to 70% facilitation) (Fig. 3). Facilitation of HCVpp-UKN1B.12.6 or HCVpp-H77 infection then disappeared at weeks 9 and 15 (i.e., HCVpp-serum incubation resulting in ca. 100% residual infectivity) (Fig. 3), when the patient elicited a neutralizing response readily detectable with HCVpp-CG1b (Fig. 1A). The concomitant development of neutralizing responses and loss of facilitation were also detected for the other patients, for example, patient Pt-6 (Fig. 3). These findings suggested that the patient sera may cross-neutralize genotypes 1a and 1b, but this neutralization can be difficult to detect depending on the overlying effect of facilitation. Thus, poor neutralization or lack of detectable neutralization in serum cannot be interpreted as absence of a neutralizing response.

Neutralizing antibody responses inversely correlate with viral replication kinetics. The neutralization and facilitation kinetics of sera from all patients included in the cohort are displayed in Fig. 1, along with HCV RNA and ALT kinetics. Overall, in one group of patients (group 1), a strong decrease in HCV replication over time correlated with the emergence of neutralizing responses (Fig. 1A), whereas a second group (group 2) did not clear or control the virus (Fig. 1B). Four patients (Pt-17, Pt-14, Pt-15, and Pt-16) (Fig. 1) could not be



B

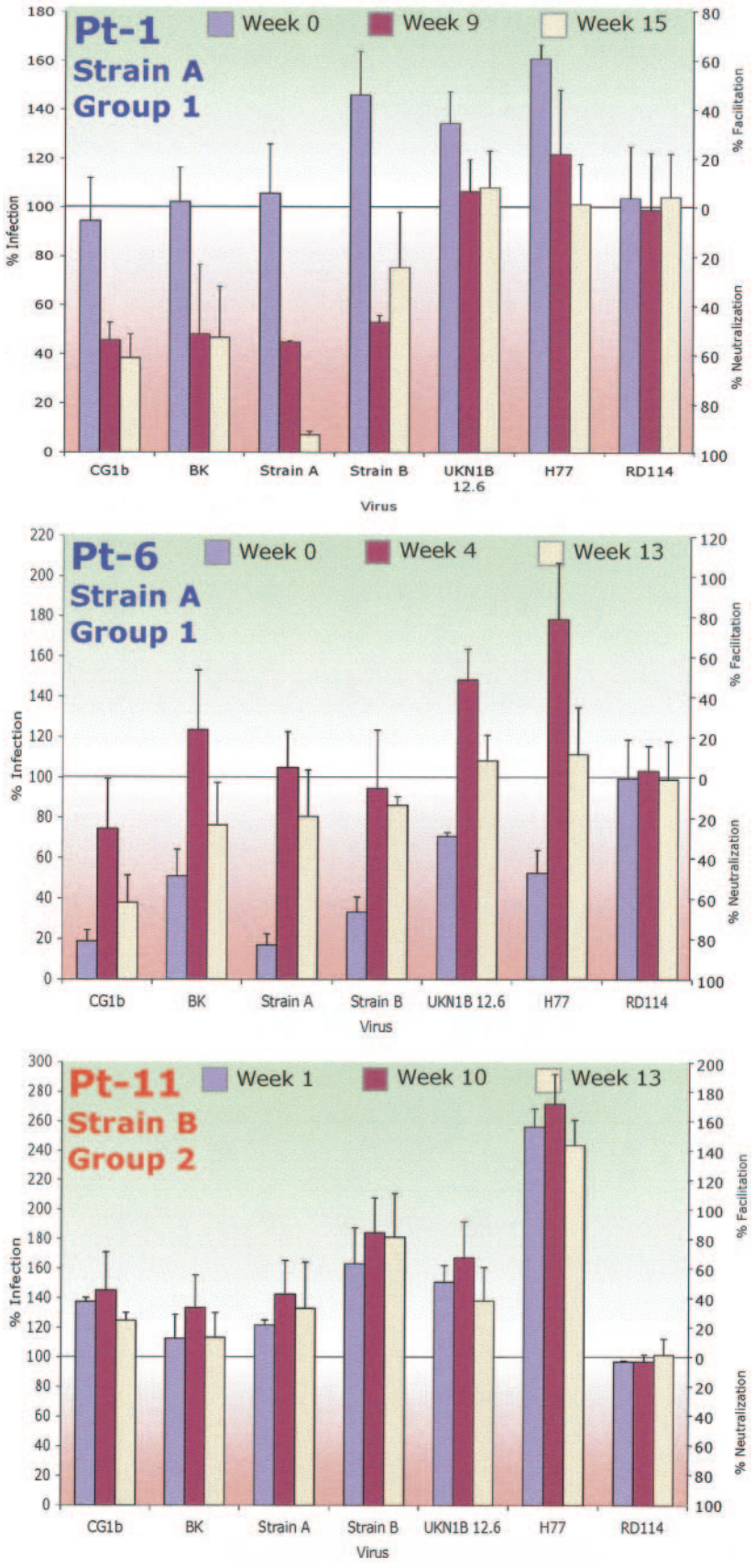
Virus strain A :	
Pt-3	TTYTTGAVQGRTLSTFTSILTRGPAQN
Pt-1	----------
Pt-2	----------
Pt-4	----------
Pt-6	----------
Pt-7	----------
Pt-8	----------
Pt-16	----------N---------
Pt-17	----------
Virus strain B :	
Pt-12	HTHVTGGSASRATRGLTALFDFGASQN
Pt-5	----------T----------
Pt-9	R----------
Pt-10	Q-Y----------
Pt-11	Q---I------N---------S-----
Pt-13	----------
Pt-15	R---------H----------

FIG. 2. HCV strain genotyping. (A) Phylogenetic analyses of full-length E1-E2 amino acid sequences from HCV strain A, infecting patient Pt-3, and HCV strain B, infecting patient Pt-12. The phylogenetic tree was constructed by the neighbor-joining method with a sequence matrix determined by a Kimura approach (PRODIST and NEIGHBOR software). The tree is artificially rooted using an HC-J6 isolate (prototype genotype 1b) as the outgroup. HCV types and subtypes are indicated above their respective branches. Only bootstrap values of >50% are indicated below the branches for 1,000 replicates. For each of the reference sequences, the accession number is given in Materials and Methods. (B) Alignment of amino acid residues of HVR1 of the HCV E2 glycoprotein. For each patient, HVR1 sequences were obtained by direct sequencing and corresponded to the major variant present in the first available serum. Amino acid sequences are shown using the one-letter code. Dashes represent residues identical to the top sequence.

classified in either of these two groups and were excluded from analysis at this stage.

Group 1. In group 1 (Fig. 1A), high viral loads ($>10^6$ i.u./ml) were detected for all patients at the time of entry into the study. These patients subsequently exhibited profound HCV RNA fluctuations that led to partial or complete control of viral replication at the end of follow-up. In this group, the patients' sera exhibited strong and specific neutralizing responses that inversely correlated with HCV RNA kinetics. Interestingly, six of these seven patients were infected with HCV strain A (Fig. 1A and 2B and data not shown). In most cases, it was difficult to precisely establish the stages of acute infection at which the patients were included in this study. Indeed, the date of infection was not precisely known. The seroconversion profiles, shown in Fig. 1A, confirmed the humoral responses to HCV infection. Antibodies were detected early during follow-up in all cases, suggesting induction of humoral responses, but different profiles of emergence of the HCV antibodies were seen in the patients. Although antibodies directed to E1 and E2 antigens were sought in order to determine the date of anti-envelope seroconversion, such antibodies could rarely be detected, probably owing to a lack of sensitivity of the line immunoassay. In acute hepatitis C, the ALT peak is concomitant with the onset of the cytotoxic cellular response. In some patients, the ALT peak was observed early during follow-up, whereas it occurred later in others, possibly suggesting earlier entry into the study for the latter. In all instances, the ALT peak preceded control of viral replication. It was not possible, however, to establish whether the control of viral replication by the host immune responses was transient or definitive (spontaneous cure of infection) in the absence of subsequent follow-up without treatment.

Whatever the stage of infection at which the patients were identified as being infected and serially sampled, the inverse relationship between HCV RNA fluctuations and neutralizing responses was obvious in all patients from group 1, as shown in Fig. 1A. Indeed, no or only low-level neutralizing responses were detected in the earliest samples from patients Pt-1, Pt-2, Pt-3, and Pt-4, who had high plateauing viremia levels. In these patients, neutralizing responses gradually increased, in parallel with the appearance of and/or increase in the titer of the other anti-HCV antibodies and a steady decrease in HCV RNA levels. The neutralizing responses then tended to decrease when HCV RNA became undetectable or very low. Patient Pt-4 was treated at the end of the follow-up period because of his youth, although his HCV RNA and ALT profiles retrospectively suggested ongoing spontaneous clearance. In contrast, patients Pt-5, Pt-6, and Pt-7 already had high levels of neutralizing responses in the earliest samples recovered for this study, probably because they had been recruited in the cohort at the later stage of acute-phase infection. In keeping with the other patients from group 1, the efficacy of neutralizing responses


Pt-1
Strain A
Group 1
Week 0
Week 9
Week 15
% Infection
% Facilitation
% Neutralization
CG1b
BK
Strain A
Strain B
UKN1B 12.6
H77
RD114
Virus
Pt-6
Strain A
Group 1
Week 0
Week 4
Week 13
Pt-11
Strain B
Group 2
Week 1
Week 10
Week 13

evolved inversely to HCV RNA fluctuations. Of note, HCV RNA was not measured for patient Pt-6 between weeks 3.5 and 7.5 (Fig. 1A), so the relationship with the neutralizing response could not be assessed during that period.

Group 2. In the second group of patients (Fig. 1B), high levels of viremia were detected in all samples from the beginning of follow-up, and they were maintained without significant fluctuations throughout the entire study period. Patients Pt-9 and Pt-11 were treated at the end of the study period because of their youth and persistently high HCV RNA levels (they received 135 μg of pegylated alpha interferon 2a per week). Interestingly, five of these six patients were infected with HCV strain B (Fig. 1A and 2B and data not shown), suggesting that infection with that strain correlated with viral persistence while infection with strain A seemed to allow a decrease of the viral load. Similarly to group 1, these patients exhibited various seroconversion profiles (Fig. 1B), and the ALT peak that witnesses the cellular response to infection was seen at various dates relative to entry into the study. Thus, this group of patients confirmed the inverse relationship between HCV RNA levels and neutralizing responses. Indeed, no or little neutralizing activity was found in the sera of these patients with sustained high-level viremia, who seemed to establish chronic infection without any control of viral replication. Importantly, incubation of HCVpp with most sera from patients from group 2 was found to facilitate infection. This is displayed for Pt-11 in the graphs in Fig. 3 by HCVpp infectivity of >100% after incubation with these sera, and consequently, as negative neutralization values in Fig. 1B.

DISCUSSION

Using a highly sensitive in vitro infection assay developed as described previously (3, 26), we found in this study that (i) human blood contains components that facilitate HCV infection; (ii) the measured neutralizing activity of serum is the result of a combination of two opposite effects, true neutralization and facilitation of infection; and (iii) the kinetics of serum neutralization inversely correlate with HCV replication kinetics in patients with acute HCV infection.

An important finding in this study is the demonstration of the capacity of human serum from infected and noninfected individuals to facilitate in vitro infection by HCV pseudoparticles generated with E1-E2 glycoproteins derived from different HCV genotype and strains, including some retrieved from our patients' sera. This suggests that a human serum component(s) may facilitate HCV infection in vivo, as is already known for several other viruses. Facilitation of HCVpp infection, however, appears to be related to the specific interaction of E1-E2 envelope glycoproteins with this component(s). Indeed, it was not observed when using pseudoparticles generated with glycoproteins derived from alternative enveloped viruses (e.g., influenza virus, vesiculovirus, and retrovirus). In addition, the degree of facilitation depended on the HCV genotype or strain (Fig. 3), i.e., on the envelope glycoprotein sequence. In this respect, it is interesting that the infectivity of HCVpp displaying the E1-E2 glycoproteins from virus strain B, which persisted in patients, was more efficiently facilitated by noninfected sera than those displaying glycoproteins from virus strain A, which was controlled during the acute phase.

The nature of the blood component(s) involved in facilitation of HCV infection remains unknown. Virus-specific antibodies have been reported to enhance viral infectivity both in vitro and in vivo (25). Indeed, viruses from various families elicit antibodies that enhance infectivity through the binding of virus-antibody complexes to cellular Fc receptors (expressed in, e.g., monocytes/macrophages) via the Fc portion of the antibodies (24, 44, 49). Fixation of the C3 or C1q complement proteins, activated by virus-antibody complexes, can also facilitate virus entry, as shown for the antibody-dependent, complement-mediated enhancement of infection of human immunodeficiency virus (19, 46, 53) and Ebola virus (54). Finally, in vitro enhancement of human immunodeficiency virus infection via an antibody-independent mechanism that involves receptors of the classical and alternative complement pathways has been reported (5, 21). However, none of these previously described mechanisms appears to be involved in the facilitation of infection by HCV pseudoparticles observed in this study, because heat-treated—decomplemented—sera from noninfected donors displayed the same levels of facilitation and because facilitation was not observed when the HCVpp were incubated with normal purified human immunoglobulin or monoclonal antibodies. On the other hand, the existence of infection-facilitating serum components is reminiscent of the fact that HCV circulates in vivo as complexes including serum proteins. Separation of infected blood samples by ultracentrifugation in sucrose or cesium chloride gradients indeed revealed two fractions in which HCV is abundant (6, 37, 60, 61). The first, at a density of ~1.25, contains viral particles mainly linked to immunoglobulins that are poorly infectious. The second, at a density of <1.06, contains viral particles that are mostly lipoprotein associated and infectious. The amount of HCV RNA in these fractions is highly variable and depends on the disease stage (60) and on the virus genotype (29). Whether the association of HCVpp with such serum components may occur in vitro upon their incubation with human serum and subsequently facilitate infection warrants further investigation.

Although definitive confirmation that human serum may facilitate HCV infection will require a reliable cell culture system to amplify wild-type virus and recover plasma-free particles, our results strongly suggest that the capacity of HCV to interact with an infection-facilitating serum factor(s) repre-

FIG. 3. Detection of HCV cross-neutralization. Pseudoparticles generated with E1-E2 glycoproteins of genotype 1b (strains CG1b, BK, and UKN1B.12.6, as well as strains A and B, respectively, derived from patients Pt-3 and Pt-11) or 1a (strain H77) or with RD114 glycoproteins were incubated with selected sera from patients Pt-1 (weeks 0, 9, and 15), Pt-6 (weeks 0, 4, and 13), and Pt-11 (weeks 1, 10, and 13). The results are expressed on the left *y* axis as percentages of the average infectious titers plus standard deviations relative to titers determined in the absence of human serum. Therefore, values of <100% represent inhibition of infectivity (red areas) (indicated on the right *y* axis as percent neutralization), whereas values over this baseline (dotted line) show facilitation of HCVpp infection by human sera (green areas) (indicated on the right *y* axis as percent facilitation). The results were derived from at least three independent experiments using different virion production batches.

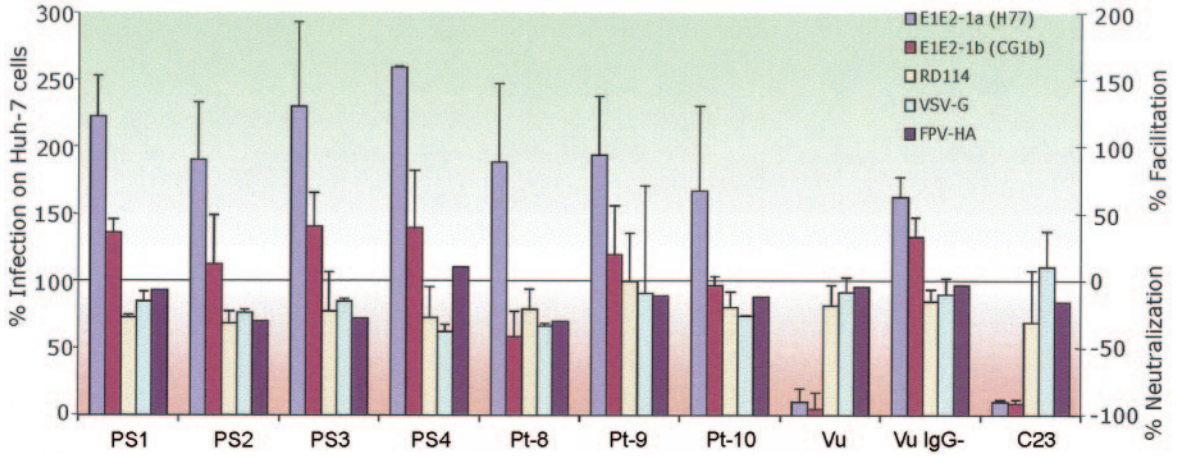


FIG. 4. Facilitation of HCV infection by sera from non-HCV-infected donors and patients from group 2. Pseudoparticles generated with E1-E2 glycoproteins of genotype 1a (strain H) or 1b (strain CG1b), as well as with RD114, VSV-G, or hemagglutinin glycoproteins (FPV-HA), were incubated with sera derived from healthy donors (PS1 to PS4) or from selected sera of patients who did not display detectable neutralizing antibodies (Pt-8 at week 12, Pt-9 at week 10, and Pt-10 at week 15). The C23 neutralizing mouse monoclonal antibody and Vu, a serum from a chronic HCV carrier, were used as positive controls, as previously described. Except for pseudoparticles generated with VSV-G, which are inhibited by human complement (50), these experiments were carried out with sera containing complement activity. Heat treatment of these sera did not eliminate the facilitation of infection (data not shown). Depletion of IgG from the sera resulted in loss of neutralization activity (Vu IgG−). The results are expressed on the left *y* axis as percentages of the average infectious titers plus standard deviations relative to titers determined in the absence of human serum. Therefore, values of <100% represent inhibition of infectivity (red areas) (indicated on the right *y* axis as percent neutralization), whereas values over this baseline (dotted line) show facilitation of HCVpp infection by human sera (green areas) (indicated on the right *y* axis as percent facilitation). The results were derived from at least three independent experiments using different virion production batches.

sents an essential component of sustained HCV infection. This not only may contribute to the "masking" of the virions from the immune system (47, 60), but may also represent a novel pathway of infection that exploits soluble serum factors for cell entry. This notion is supported first by our observation that facilitation of infection correlated with sustained high replication levels in the patients from group 2. Our data also suggest that the rise in neutralizing responses during the course of infection is associated with the disappearance of serum-facilitating effects, as is clearly seen in patients from group 2, such as Pt-8, Pt-9, and Pt-10. Whether facilitation of infection is eliminated in these cases or is still there but is overwhelmed by a counteracting neutralizing force remains to be determined.

The second important finding in our study is the inverse relationship between HCV RNA levels and the level of neutralizing responses in patients' sera. This inverse relationship was observed in the two groups of patients, including patients who underwent profound HCV RNA fluctuations and ultimately evolved toward HCV RNA clearance from serum (group 1) and patients who maintained high replication levels throughout the entire follow-up period (group 2). Patients from group 1 indeed elicited a neutralizing response that inversely followed HCV RNA kinetics, whereas the patients from group 2 remained with undetectable neutralizing response and exhibited a facilitating effect of their sera on HCVpp infectivity. The reason why the latter group of patients failed to raise a neutralizing response is unclear. It is not related to their inability to induce a humoral immune response, since these patients seroconverted during the study period for the same antibodies and with the same amplitude as the patients from group 1. The difference could eventually be explained by the fact that a majority of the two groups of patients were infected by either of two distinct HCV strains. This might suggest that different viruses bear intrinsic properties influencing their abilities to raise a neutralizing response upon infection. In this respect, chimpanzee challenge experiments with the two HCV strains from this study would be of major interest.

The observation of an inverse relationship between HCV RNA and neutralizing response kinetics at the acute phase of infection in our hemodialysis patients should be interpreted cautiously in a context where the role of humoral responses in the control of HCV infection is largely unknown. While vigorous and broad T-cell responses are involved in viral clearance (40), a role for antibodies in protection against natural HCV infection has been difficult to establish. Nevertheless, there is evidence that polyclonal antibodies to HCV can be protective. Immunoglobulin preparations manufactured before the screening of plasma donors for HCV became common practice were shown to protect recipients against HCV infection (17, 62). Indeed, broadly reactive neutralizing and protecting antibodies were found in experimental immune globulin preparations made from anti-HCV-positive donations, as well as in a commercial immune globulin product, Gammagard, prepared from unscreened plasma (62). Subsequent products prepared from pooled plasmas from which anti-HCV-positive donations had been excluded were reported to transmit HCV to recipients, potentially as a result of the removal of neutralizing antibodies (62). In addition, studies of chimpanzees have also shown that anti-HCV immunoglobulins or hyperimmune sera can delay or prevent infection when the virus is inoculated after or at the same time as the antibodies (16, 30). However, whether a neutralizing response could be elicited at the acute phase of natural HCV infection and the precise role of neutralizing antibodies in the transient or sustained control of viral replication during acute hepatitis C remain unclear. In experimentally inoculated chimpanzees, Logvinoff et al. (35) found that none of three animals with acute resolving infection developed neutralizing antibodies and, for other animals, that such

antibodies could be detected in sera of acutely infected animals who did not resolve infection. This suggested that neutralizing antibodies did not play a critical role in the resolution of acute HCV infection, at least in the chimpanzee model. In contrast, our study shows for the first time the early emergence of a neutralizing response in patients who apparently evolved toward a control of viral replication, with strong responses concomitant with steep HCV RNA decreases (>4 log units) together with ALT normalization. The correlation of a relatively strong neutralizing response with a substantial loss of viremia was corroborated by the observation that, in the second group of patients, failure to reduce HCV RNA levels was associated with a lack of detection of a neutralizing response in blood from these patients.

At the present time, however, it is not possible to establish whether HCV RNA drops were actually the unequivocal consequence of the increase in neutralizing responses. In other words, whether neutralizing responses played a major role in the control of viral replication in group 1 patients is unclear. The fact is, however, that only the patients who could mount an efficient neutralizing response were able to control viral replication, whereas those who had no detectable neutralizing response continued to display very high levels of viral replication. Neutralizing antibodies alone do not appear to be able to control viral replication and lead to definitive HCV clearance. Indeed, cases of spontaneous resolution have been reported in HCV-infected agammaglobulinemic children (1, 4, 8), suggesting that control of HCV may occur independently of antibodies, at least in a limited number of patients. Furthermore, it has been shown that chimpanzees vaccinated with recombinant HCV glycoproteins that induced high-titer antibodies were partially protected against a subsequent low-dose homologous HCV challenge (7). However, experimentally infected chimpanzees and naturally infected humans could be reinfected with homologous and heterologous HCV strains, suggesting that humoral immunity that develops after spontaneous resolution of acute hepatitis C is not sterilizing (15, 32, 45). The role of escape mutation selection, particularly in the HVR1 region, in the establishment of chronic infection has been suggested, both at the acute phase of infection and in patients receiving alpha interferon-based antiviral therapy (13, 43), a hypothesis that will be tested in the present series of patients. At this stage, our preliminary observation of an inverse relationship between HCV RNA and neutralizing response kinetics in acutely infected patients is challenging, yet further characterization of neutralization responses during acute hepatitis C is crucial to understanding HCV pathogenesis and developing efficient vaccines.

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ADDENDUM IN PROOF

Since this paper was submitted, we have identified the facilitating serum component as high density lipoprotein (HDL; 11th International Symposium on Hepatitis C Virus and Related Viruses, Heidelberg, Germany, October 2004). At physiological concentrations, HDL facilitates HCVpp infection as efficiently as human serum. HDL enhances HCVpp infection in an interplay with the hypervariable region 1 of the E2 glycoprotein and the scavenger receptor B1 and renders HCVpp less sensitive to neutralizing antibodies (B. Bartosch et al., submitted for publication).

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