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Reduction of Hepatitis C Virus Using Lectin Affinity Plasmapheresis in Dialysis Patients

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Key Words
Hepatitis C virus · Lectin affinity · Hemodialysis · *Galanthus nivalis* agglutinin

Abstract
Background/Aims: To test the safety and efficacy of the Aethlon Hemopurifier®, a lectin affinity cartridge, in clearing hepatitis C virus (HCV) from the blood of HCV-positive end-stage renal disease patients undergoing dialysis. Viral RNA was measured using real-time quantitative reverse transcriptase polymerase chain reaction. ***Results:*** HCV clearance from plasma or blood was measured using either direct capture on immobilized *Galanthus nivalis* agglutinin (GNA) or using miniature plasmapheresis cartridges containing immobilized GNA. HCV in plasma samples was rapidly cleared by direct affinity capture ($t_{1/2}$ = approx. 20 min) and HCV in human blood was cleared using the Hemopurifier ($t_{1/2}$ = 2–3 h). Institutional-review-board-sanctioned clinical safety studies were conducted at the Apollo and Fortis Hospitals in India. At Apollo, 4 patients were treated 3 times/week for 2 weeks. HCV captured on the Hemopurifier averaged 8.9×10^8 viral copies/cartridge (n = 5), representing approximately 30% of the initial viral body burden. At Fortis, 3 patients treated 3 times/week for 1 week completed the viral load studies. Two patients showed measurable viral load reduction, while the third showed both increases and decreases in viral load. After Hemopurifier treatment, average HCV viral load was reduced by 57%. Surprisingly, average viral load was also 82% lower 7 days after treatment. Control samples also showed a marked transient reduction in HCV viral load as previously reported. ***Conclusion:*** The Hemopurifier rapidly cleared HCV from blood treated in vitro. In patients, the combination of the Hemopurifier plus dialysis decreased HCV viral load by 57% in 1 week. Moreover, viral load reduction continued up to 7 days after treatment.

Introduction

Hepatitis C virus (HCV) is the most common chronic blood-borne infection in the United States. The Third National Health and Nutrition Examination Survey (1999) estimates that 3.9 million US citizens have been infected with HCV [1]. Worldwide over 170 million persons are HCV infected [2]. In Western Europe, HCV is the leading cause of liver disease and the most common reason for liver transplant.

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HCV is a flavivirus that primarily infects hepatocytes and only grows in humans and chimpanzees. The virus is an enveloped, roughly spherical 50-nm particle containing a single positive-strand RNA genome of 9.6 kb. The viral envelope contains two surface glycoproteins E1 and E2 that are involved in receptor binding. E1 and E2 are type I integral transmembrane proteins, with C-terminal hydrophobic anchor domains.

HCV is poorly controlled by natural immunity. HCV is highly mutable, producing quasi-species that have the potential to be selected for drug resistance [2, 3]. HCV exists in at least 6 distinct HCV genotypes [4, 5]. Genotype 1 is the most common in the United States. Patients with genotypes 2 and 3 are almost three times more likely to respond to therapy than patients with genotype 1.

We have been investigating the use of lectin affinity capture to reduce circulating HCV. *Galanthus nivalis* agglutinin (GNA) binds HCV envelope glycoproteins with high specificity [6, 7] and has been used in ELISA to assay E2 [8]. The evidence suggests that the envelope glycoproteins of HCV are of the high mannose type and similar to polysaccharides found on many other viral envelope glycoproteins (e.g. HIV gp120).

Prior studies have shown that hemodialysis with a cellulosic or synthetic dialyzer reduces HCV viral load by 3–95% [9]. Fernandez et al. [10] report that HCV viral load in dialysis patients could be reduced by 40% in one session, while Mizuno et al. [11] report that HCV viral load reduction during hemodialysis is membrane dependent. These authors found that HCV viral load dropped 10–100 times after dialysis on polysulfone membrane. In contrast, AN69 membrane only worked in a subset of patients, while Cuprophan-based hollow fibers had no effect. Fabrizi et al. [12] report that HCV-positive dialysis patients showed no significant viral load reduction over a period of 13 months.

On the basis of these reports, normal hemodialysis seems to be capable of transiently reducing viral load, dependent in part on the type of hollow fiber membrane used. This correlates with results obtained by Ramratnam et al. [13] where therapeutic plasma exchange caused only transient alterations in HCV or HIV viral load, followed by return to baseline in 3–4 h. In this study, HCV viral load was reduced to 50% of its initial value followed by return to baseline in 3–4 h. Thus, the degree of HCV removal during dialysis remains debatable.

We have been testing the ability of GNA lectin plasmapheresis devices (Hemopurifier® cartridges) to clear HCV from the blood of HCV-positive end-stage renal disease (ESRD) patients undergoing dialysis with the goal of developing a therapeutic device that can be used as adjunctive treatment for chronic and acute viral infections. The studies reported here are the first human studies with these devices.

Materials and Methods

Materials

GNA and GNA agarose were obtained from Vector Labs (Burlingame, Calif., USA). HCV-infected plasma samples were obtained from Boston Biomedica, Inc. (Boston, Mass., USA), Zeptometrix (Buffalo, N.Y., USA) and from Accudx (San Diego, Calif., USA). Normal human plasma and blood were obtained from the San Diego Blood Bank (San Diego, Calif., USA). PCR primers KY78 (HCV reverse primer: CTC GCA AGC ACC CTA TCA GGC AGT) and KY80 (HCV forward primer: GCA GAA AGC GTC TAG CCA TGG CGT) were obtained from Operon (Alameda, Calif., USA). Linear polyacrylamide (PC152) was purchased from Molecular Research Center. All other reagents used were reagent grade or better.

Isolation of HIV Viral RNA

For blood samples, plasma was isolated by low-speed centrifugation. Viral RNA was prepared from plasma using TRI-LS reagent (MRC) essentially as follows: 250 μl of plasma was mixed with 750 μl TRI reagent and allowed to stand 30 min followed by centrifugation (10 min at 12,000 rpm) in the cold. The upper phase containing the RNA was carefully removed to avoid contamination with DNA and proteins in the interphase and the RNA precipitated with 1 volume cold isopropanol with 2 μl/ml linear polyacrylamide carrier. The RNA was pelleted for 10 min at 13,000 rpm, triturated with 75% ethanol, air dried and dissolved in 10–25 μl RNase-free water.

Measurement of Viral RNA

Viral RNA was measured by real-time quantitative reverse transcriptase polymerase chain reaction performed using an Access RT-PCR reagent set (Promega, Madison, Wisc., USA) in 25 μl reaction volumes containing 0.2 units/μl each of Tfl DNA polymerase and AMV reverse transcriptase, 0.2 μM KY78 and KY80 primers, SYBR green (1:10,000), 1 × SCA blocking mix (Cepheid), 2.5 mM $MgSO_4$, 400 μM dNTPs and 5 μl of unknown RNA or HCV armored RNA standard (Ambion, Austin, Tex., USA). Amplification and reaction times were: reverse transcriptase (45 min at 42°C) and polymerase chain reaction 40 cycles (94°C/30 s; 55°C/30 s; 72°C/60 s; 87°C/6 s) read in a SmartCycler real-time thermocycler (Cepheid, Sunnyvale, Calif., USA) essentially according to the manufacturer's instructions. For clinical studies, viral load was determined in duplicate or triplicate at Arbropharma (New Delhi, India).

Removal of HCV by Direct Immunochromatography

Small-volume filter cartridges (Glen Research, Sterling, Va., USA) containing 0.6 ml affinity resin were equilibrated with 5–10 column volumes of sterile phosphate-buffered saline (PBS). Plasma of 1.5–3 ml containing HCV (typically 10^6 cpm) was circu-

Table 1. HCV captured on the Hemopurifier during treatment

Patient	Cart. No.	Average capture		Initial plasma viral load		Capture %
		HCV copies	±2 SEM	HCV copies	±2 SEM	
1	H6	1.99×10^9	8.4%	3.80×10^9	17%	52
2	H1	2.25×10^8	35%	2.49×10^9	10%	9
	H6	1.19×10^8				5
3	H1	5.15×10^8	41%	1.60×10^9	22%	32
	H6	5.01×10^8				31
4	n.a.	n.a.		n.a.		n.a.
Average		8.89×10^8		2.63×10^9		30

lated over the column at a flow rate of 0.5–0.6 ml/min at room temperature. The circulating plasma was tested at intervals for the presence of HCV RNA.

Preparation and Testing of Affinity Hemodialysis Cartridges

The GNA Hemopurifier was made by introducing a slurry of GNA agarose beads or GNA coupled to celite in sterile PBS buffer into the outside compartment of a hollow-fiber dialysis column via syringe. For small-scale tests we used Microkros polyethersulfone hollow-fiber plasmapheresis cartridge equipped with Luer fittings (200 μm inside diameter × 240 μm outside diameter, pore diameter 200–500 nm, approx. 0.6 ml internal volume) obtained from Spectrum Labs (Rancho Dominguez, Calif., USA). Cartridges containing the affinity resin were equilibrated with 5–10 column volumes of sterile PBS. HCV-infected blood samples were prepared by mixing 5 ml type O+ fresh packed red cells with 5 ml HCV-infected plasma (approx. 10^6 cpm). The blood was recirculated over the column at 0.5–0.9 ml/min at 37°C using a Masterflex roller pump (1 rpm) and Pharmed 6485-16 tubing.

For patient studies, the GNA Hemopurifier was prepared using a Plasmart PS-60 plasma separator (Medica srl, Medollo, Italy) containing GNA immobilized on Chromasorb GAW 60/80 (Celite Corp., Lompoc, Calif., USA) manufactured under good manufacturing practices. The products were packaged and gamma sterilized (approx. 33 kGy). Sterility and endotoxin levels were determined to be within acceptable limits (WuXi Apptech, Marrietta, Ga., USA). Bench studies showed no detectable leaching of either silica or GNA. ISO10993 biocompatibility studies of the Hemopurifier performed at the Shriram Institute (New Delhi) did not cause detectable hemolysis, cytotoxicity, complement generation, skin sensitization, intracutaneous or acute systemic toxicity in animal test models.

Clinical Safety and Preliminary Efficacy Studies

Institutional review board approval was obtained for two safety study protocols for two different hospitals in New Delhi. At the Indrapratha Apollo Hospital, the safety study was for 4 HCV-positive ESRD patients treated with the GNA Hemopurifier in conjunction with normal hemodialysis for 4 h 3 times a week for 2 weeks. The second study at the Fortis Hospital involved 6 HCV-positive ESRD patients treated with the GNA Hemopurifier in conjunction with normal hemodialysis for 4 h 3 times a week for 1 week. The latter study is currently ongoing. The details of these studies will be presented elsewhere.

Results and Discussion

Initial experiments measured the efficiency of native HCV capture from plasma and blood. HCV was rapidly cleared from plasma when passed directly over the GNA affinity matrix. The estimated clearance half-time was 15–20 min at room temperature. In a second set of experiments, HCV clearance in blood was measured using the GNA Hemopurifier. The results indicate a substantial capture of HCV in less than 6 h. The estimated half-time for clearance was approximately 2 h. The observed clearance rates are very similar to those observed previously for HIV-infected plasma [14] using an HIV-specific antibody as the capture agent and in subsequent studies using immobilized GNA [unpubl. obs.].

In subsequent studies, we observed some HCV samples that did not bind well to the GNA agarose. We obtained a low-binding HCV sample from Zeptometrix and recirculated it over GNA agarose. Aliquots taken at various time intervals were then fractionated by equilibrium CsCl density gradient ultracentrifugation into low- and high-density fractions. The high-density HCV fraction was 70% cleared in 3 h. The low-density fraction was not captured to a measurable degree.

Literature reports indicate that HCV can associate with lipoproteins [15]. In some HCV plasma samples 95% of the virus forms a low-density particle about twice the size of HCV alone. Our results indicate that low-density virus particles do not bind well to GNA lectin. It is therefore reasonable to presume that these low-density nonbinding virus particles are lipoprotein associated. Furthermore, it is likely that patient samples will show a variable degree of HCV capture on GNA affinity matrices due to low-density lipoprotein content variation.

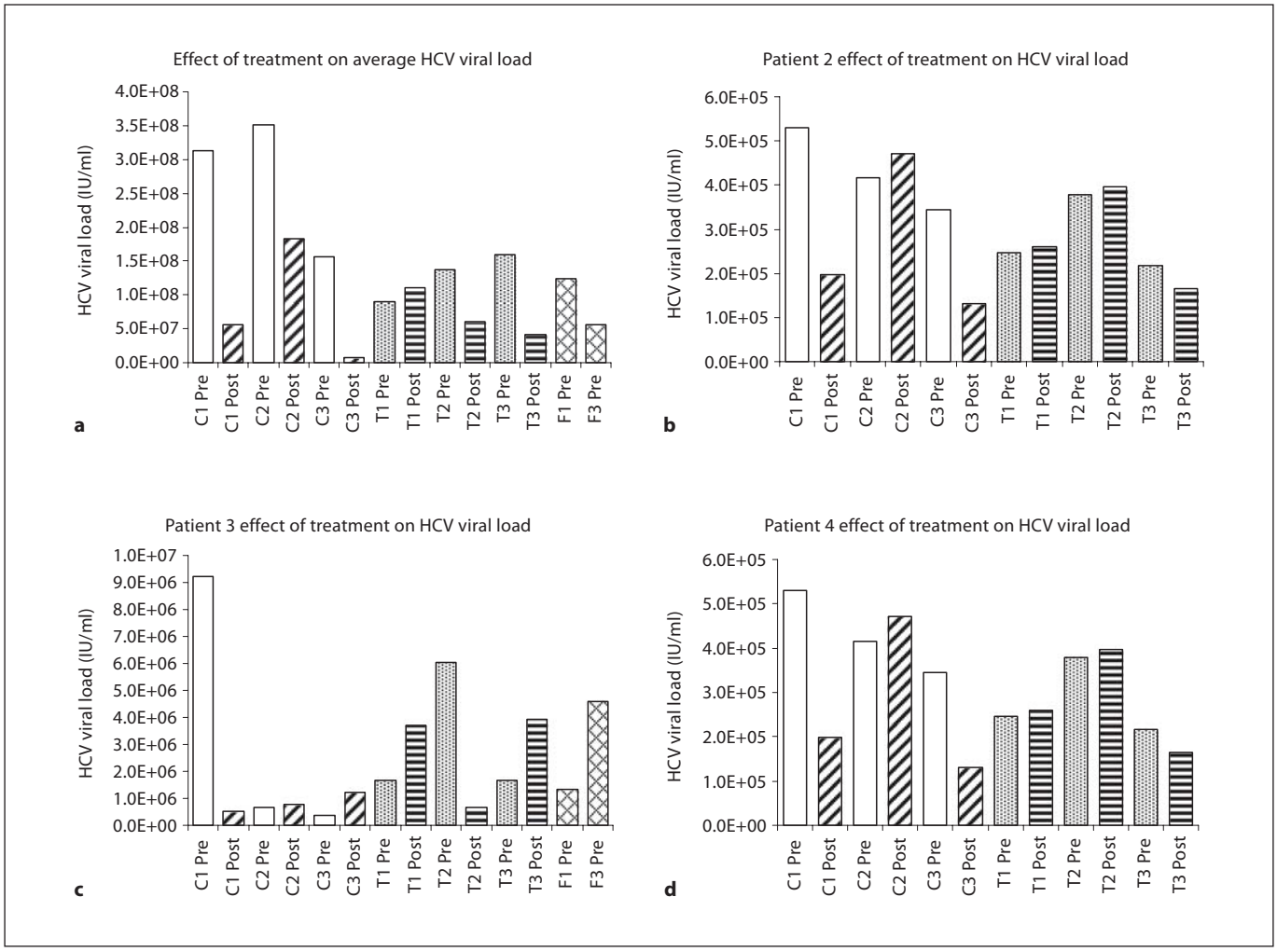

Fig. 1. HCV viral load from dialysis patients during a clinical safety study on HCV-infected ESRD patients. Each treatment lasted 4 h, repeated 3 times a week. Control was dialysis alone for 1 week using a fresh Fresenius F80 dialyzer for each session [C1–C3 before (Pre) and after (Post) dialysis]. The treatment [T1–T3 before (Pre) and after (Post) dialysis] included the Hemopurifier in series with the F80 dialyzer for 1 week. Follow-up data (F1 Pre and F3 Pre) were taken prior to the normal dialysis sessions on day 3 and 7 after the last Hemopurifier treatment. Blood flow rate = 300 ml/min. Effect of treatment on HCV viral load: average (**a**), patient 2 (**b**), patient 3 (**c**), patient 4 (**d**).

Preliminary Efficacy Determination

The Apollo Hospital studies provided some basic information on HCV capture. In these studies, the ability of the GNA Hemopurifier to capture HCV was assessed by measuring HCV bound to the GNA Hemopurifier cartridge for 3 of the 4 enrolled subjects. As shown in table 1, HCV capture ranged from 1.19×10^8 to 1.99×10^9 viral copies. These values represented clearance of 5–52% of the estimated initial circulating body burden of HCV. Average clearance was 7% (patient 2), 32% (patient 3) and 52% (patient 1). Overall, the average HCV capture represented 30% of the initial burden of HCV in plasma. It is possible that patient-to-patient variability may represent variations in lipoprotein association.

Subsequent studies performed at the Fortis Hospital looked at plasma viral load before and after the control (dialysis only) or experiment (GNA Hemopurifier plus dialysis). Figure 1 shows HCV viral load before and after each treatment as the overall average for all patients and for each patient individually. The results were surprising in the extent to which they confirm that control dialysis alone produced a transient HCV viral load reduction and rebound.

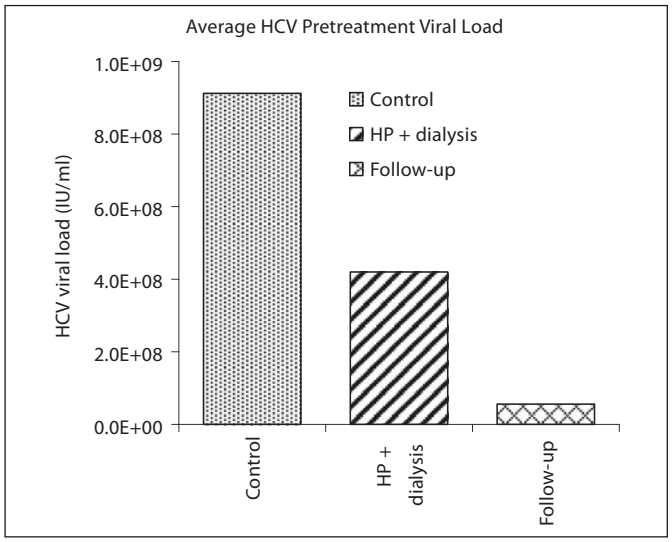

Fig. 2. Average HCV viral load in patients prior to treatment comparing control dialysis to Hemopurifier + dialysis and final values in the week after treatment was stopped. Final values represent the average of HCV viral load for the week after treatment was stopped (follow-up phase).

This is most easily seen in figure 2 which compares the average HCV viral load just prior to the start of the procedure for each treatment stage (control dialysis, Hemopurifier plus dialysis, and follow-up). Compared to the control dialysis stage, the Hemopurifier plus dialysis HCV viral load is significantly and clearly reduced. Perhaps more interesting is that the HCV viral load reduction continues into the follow-up stage (days 3 and 7 following the end of the Hemopurifier treatment). This result suggests that the viral load reduction is more robust than should be expected based simply on the net replication rate of the virus [13]. Since clearance rates tend to remain relatively constant, the simplest interpretation for this result is that either the number of infected cells or the rate of viral replication must have declined.

Conclusions

Bench studies show that the GNA Hemopurifier clears HCV efficiently from both plasma and blood. However, HCV appears to associate with lipoproteins that can prevent binding to the GNA matrix to varying degrees. In clinical studies at the Apollo Hospital, the Hemopurifier effectively removed up to 1.2×10^9 copies of HCV from patients in 4 h. The Fortis Hospital study shows that the Hemopurifier and hemodialysis can clear HCV. Viral load reduction is presumably due in part to membrane binding. However, the combination of the Hemopurifier with dialysis caused a reduction in HCV viral load that continued for 7 days after the Hemopurifier treatment was stopped. The overall reduction in viral load was 55% at the end of treatment and 82% after 7 days of follow-up. This suggests a robust reduction in the number of HCV-infected cells or perhaps less likely in the rate of HCV viral replication. Additional studies are planned to extend treatment up to 30 days and optimize treatment schedules in both HCV and HIV patients.

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