Partitioning of hepatitis C virus during Cohn-Oncley fractionation of plasma

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Because of concern about the safety of immune globulins with respect to transmission of hepatitis C, the partitioning of hepatitis C virus (HCV) during alcohol fractionation of a plasma pool prepared exclusively from anti-HCV-reactive donations was examined. Quantitation of HCV RNA was accomplished by nested polymerase chain reaction (PCR) at limiting dilutions. One PCR unit was arbitrarily defined as the minimum amount of HCV RNA from which an amplified product could be detected. The starting plasma pool contained 1.4×10^5 PCR units per mL. Most of the HCV RNA was found in cryoprecipitate and in Cohn fractions I and III, but it was also detected in fraction II, which is used for immunoglobulin G preparations. A 3.4-percent solution of IgG prepared from this fraction II contained 30 PCR units per mL. The fractionation process leading to immune globulin resulted in overall reduction in HCV RNA by a factor of 4.7×10^4. Although the presence of HCV RNA in the final product does not necessarily imply the presence of infectious virus, this work suggests that the safety of immune globulins with respect to HCV transmission is not due solely to the partitioning of HCV away from the immunoglobulin fraction. **TRANSFUSION** 1992;32:824–828.

Abbreviations: HCV = hepatitis C virus; PCR = polymerase chain reaction; RTR = reverse transcriptase reaction.

HEPATITIS C VIRUS (HCV) has been identified as a major cause of non-A,non-B hepatitis associated with blood transfusion.[1-5] The cloning of a portion of the genome of this virus[6] led to the development of a test for the detection of antibodies directed against a nonstructural component of the virus,[1] and it has been shown that this test will detect a substantial proportion of infectious blood donations.[5] Anti-HCV testing is presently applied to whole blood and all transfusible components. However, until recently, the application of this test to plasma intended only for further manufacture into injectable products was discouraged by the United States Food and Drug Administration. This decision was based upon uncertainty about the effect such screening might have upon the concentration of HCV in plasma pools and about the contribution that antibodies to HCV might make to the safety of plasma derivatives, particularly immune globulin products.[7]

In recent years, several reports of non-A,non-B hepatitis attributed to intravenous immune globulin have appeared.[8-11] Although US-licensed immune globulin preparations have not been implicated in hepatitis transmission, the reasons for their apparent safety are unclear. These preparations are made from large pools of plasma (undoubtedly containing HCV), and, in most cases, no deliberate virucidal step is employed in their manufacture. HCV may be removed and/or inactivated during the fractionation process. It is also possible that small amounts of HCV contaminating the immune globulin fraction might be neutralized by antibodies present in that fraction.

Recently, a number of investigators have applied the polymerase chain reaction (PCR) assay to the detection and quantitation of HCV RNA in biologic fluids[12-16] and in certain plasma derivatives.[17,18] With a nested PCR, in which two sets of primers are used in sequence, a single copy of template cDNA can be amplified to such an extent that the product is readily detected by ethidium bromide staining.[12] Time-consuming blotting and autoradiographic steps are thus unnecessary.[12,18] The quantitation of HCV RNA can be achieved by limiting-dilution analysis.[19]

The present study describes the use of nested PCR to examine the partitioning of HCV RNA during the fractionation of plasma to produce immune globulin. In addition, the effect of screening for anti-HCV upon the viral load of plasma pools was investigated.

Materials and Methods

Plasma collection and testing

The plasma used in these experiments was collected at plasmapheresis centers operated by Alpha Therapeutic Corporation (Los Angeles, CA), Baxter Healthcare Corporation (Deerfield, IL), Community Blood and Plasma Corporation (Rochester, MI), Plasma Alliance, Inc. (Knoxville, TN), and Miles, Inc. (Berkeley, CA). Donors met all current Food and Drug Administration and industry requirements for source plasma donors;

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each donation was negative for hepatitis B surface antigen and nonreactive for antibody to human immunodeficiency virus type 1, and each unit had an alanine aminotransferase level less than twice the upper limit of normal. A total of 3073 acceptable units were collected. Each of these units was then tested with the HCV enzyme-linked immunosorbent assay (Ortho Diagnostics Systems, Inc., Raritan, NJ) according to the manufacturer's instructions; 186 units (6.1%) were repeatably reactive for anti-HCV. An anti-HCV-negative pool was prepared by combining 2-mL samples from each of the 2887 units that did not react for anti-HCV, and an anti-HCV-reactive pool was similarly prepared from 2-mL portions of the 186 anti-HCV-reactive units. Both of these pools were stored below −18°C until used.

Fractionation of anti-HCV-reactive plasma

We performed plasma fractionation by the process used by commercial fractionators, namely methods 6 and 9, respectively, of Cohn et al.[20] and Oncley et al.,[21] modified as necessary for laboratory scale. Samples for PCR were taken at various stages as indicated (Fig. 1) and were stored at −40°C until assayed. We carried out fractionation in a thermally insulated, 200-mL, stainless steel beaker and accomplished cooling and stirring by means of a magnetic stirrer and a cold plate. The frozen, anti-HCV-reactive pool was thawed and brought to 0°C, and then 100 mL was centrifuged (25 min, 16,000 × *g*) to collect the cryoprecipitate (Sample 1). We decanted the supernatant liquid (Sample 2), adjusted it to pH 7.04, and then added 95-percent ethanol to 8 percent (vol/vol), while the mixture was stirred and cooled to −3°C. The suspension was held at this temperature for 30 minutes and then centrifuged (25 min, 16,000 × *g*, −3°C) to remove fraction I (Sample 3). We adjusted the supernatant I (Sample 4) to pH 6.76, 21-percent ethanol at −5°C; it was stirred for 17 hours and then the mixture was centrifuged (25 min, 16,000 × *g*, −5°C) and the supernatant II + III decanted (Sample 5). The precipitate (fraction II + III) was suspended in 70 mL of ice water and stirred at 0 to 2°C for 1.5 hours (Sample 6); then the pH was adjusted to 6.68, and 95-percent ethanol was added to 20 percent while cooling to −5°C. After 1 hour, we centrifuged the mixture as above. The supernatant II + IIIw was decanted (Sample 7); the precipitate (fraction II + IIIw) was suspended in 94 mL of ice water, stirred for 30 minutes at 0°C, and then adjusted to pH 5.21 (Sample 8). We added ethanol to 17 percent while the mixture was cooled to −5°C; after it was stirred for 1 hour, we centrifuged the mixture as before. The fraction III precipitate (Sample 9) was harvested, supernatant III (Sample 10), at −5°C, was adjusted to pH 7.38, and ethanol was added to increase the alcohol concentration to 25 percent. After 1-hour stirring at −5°C, we centrifuged the mixture as before. The supernatant II (Sample 12) was decanted and the fraction II precipitate (Sample 11), 2.15 g, was stored at −40°C. We dissolved a 1.08-g portion of fraction II in 4 mL of 0.01 *M* (0.01 mol/L) sodium phosphate and 0.145 *M* (0.145 mol/L) NaCl, pH 7.4, and dialyzed it for 18 hours at 5°C against phosphate-buffered saline to remove residual ethanol. After dialysis, this solution of IgG, at 34.1 mg of protein per mL, was frozen at −40°C until analysis was performed (Sample 13).

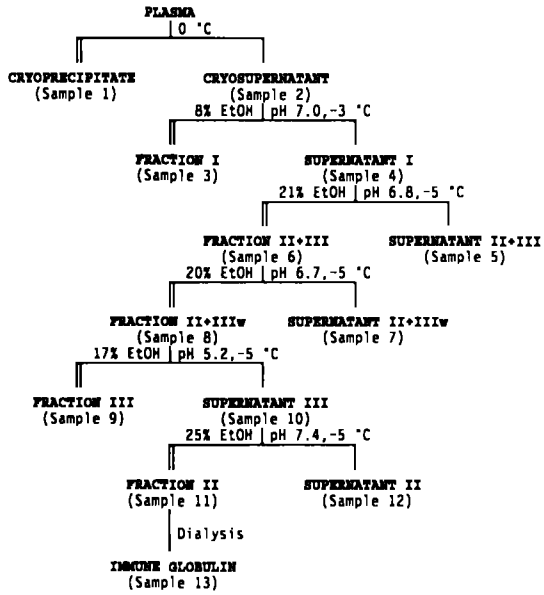

FIG. 1. Schematic diagram of the Cohn-Oncley fractionation process as employed in this study. The ethanol concentration (EtOH), pH, and temperature for each precipitation step are indicated. Sample numbers correspond to those given in Materials and Methods and in Fig. 2 and Table 2.

Primers

All four primer sequences (5′→3′) were derived from the 5′-noncoding region of the HCV genome.[22] We used Primer 1, ACTCCACCATAGATCACTCC (nts 07-26), and Primer 2, GGTGCACGGTCTACGAGACCT (nts 304-324, reverse polarity), for the cDNA synthesis and the first PCR. Primer 3, ACTCCCCTGTGAGGAACTACT (nts 22-42), and Primer 4, AACACTACTCGGCTAGCAGT (nts 229-248, reverse polarity), were the inner primers nested within the outer pair and were used for the second PCR.

RNA extraction

We precipitated RNA from samples of plasma or plasma fraction by treatment with polyethylene glycol compound (MW 15,000-20,000; Sigma Chemical Co., St. Louis, MO) to a final concentration of 8 percent and held it at 4°C overnight. The pellet, obtained by centrifuging at 12,000 × *g* for 10 minutes at 4°C, was dissolved in 0.5 mL of denaturing solution D (4 *M* [4 mol/L] guanidine thiocyanate, 0.5% N-lauroylsarcosine, 25 m*M* [25 mmol/L] sodium citrate, 0.1 *M* [0.1 mol/L] 2-mercaptoethanol, pH 7.0) and extracted with phenol-chloroform essentially as described previously.[23] The RNA present in the aqueous phase was precipitated with 2-propanol at −70°C in the presence of carrier sheep fibroblast tRNA (Sigma). We harvested the precipitate by centrifugation, washed it with 70-percent ethanol, and air-dried and redissolved it in 100 μL of diethyl pyrocarbonate-treated water supplemented with 20 U of recombinant ribonuclease inhibitor (RNasin, Promega, Madison, WI). We prepared serial dilutions of this solution and carried out reverse transcription on 50-μL portions of these dilutions.

RNA PCR

We carried out the reverse transcriptase reaction (RTR) and PCR with a thermal cycler (Geneamp System 9600, Perkin-Elmer, Norwalk, CT). All experiments included HCV-positive (H strain) and -negative control plasmas. The RTR and first PCR were performed in a 100-μL reaction volume containing 50 μL of the RNA dilution, 2.5 U of recombinant *Taq* DNA polymerase (Perkin-Elmer), 8 U of avian myeloblastosis virus

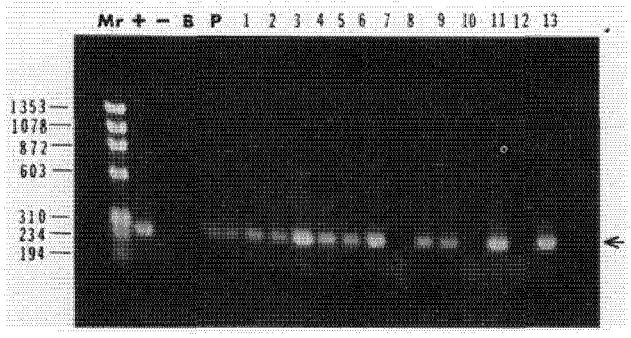

FIG. 2. Detection of HCV RNA in plasma fractions by PCR. PCR-amplified HCV RNA products were analyzed on a 1.2-percent agarose gel stained with ethidium bromide and illuminated by ultraviolet light. The DNA size marker (Mr) is *Hae* III-digested φX174 RF DNA. The arrow indicates the detected PCR product, 227 base pairs in size. + positive control plasma (H strain); −, negative control; B, blank; P, anti-HCV-reactive plasma pool; Samples 1 through 13, samples from fractionation of anti-HCV-reactive pool with sample numbers corresponding to those given in Fig. 1 and Table 2.

reverse transcriptase (Promega), 16 U of RNasin, 100 μM each of four deoxyribonucleoside triphosphates, 0.2 μM each of the first primer pairs, and 50 m*M* (50 mmol/L) KCl, 1.5 m*M* (1.5 mmol/L) $MgCl_2$, 0.01-percent gelatin, and 10 m*M* (10 mmol/L) Tris-HCl, pH 8.3 (GeneAmp DNA amplification reagent; Perkin-Elmer). The RTR was performed at 42°C for 15 minutes and then for 3 minutes at 94°C to denature the avian myeloblastosis virus reverse transcriptase activity. We subsequently performed the PCR in the same tube for 25 cycles, each cycle consisting of 45 seconds at 94°C (denaturing), 45 seconds at 55°C (annealing), and 50 seconds at 72°C (extension). We subjected 10 μL of the first PCR product to an additional 25 cycles of amplification with the second set of primers, using the same ingredients and conditions as described above, except that we excluded the avian myeloblastosis virus reverse transcriptase and RNasin. The second PCR products (10 μL) were analyzed by electrophoresis in 1.2-percent agarose gel at 60 V for 1.5 hours, and amplified DNA species were visualized under ultraviolet light after being stained with ethidium bromide.

To estimate HCV RNA concentrations of samples, we subjected serial $10^{0.5}$-fold (3.16-fold) dilutions of the RNA extracts to RTR and PCR. One PCR unit was arbitrarily defined as the minimum amount of HCV RNA from which an amplified product could be visualized. Inasmuch as the PCR procedure is capable of detecting a single template of cDNA,[12,18] the number of PCR units in a sample is approximately equal to the number of copies of HCV RNA in the sample multiplied by the efficiency of the RTR, which has been estimated to be about 5 percent.[15] When all of the replicates at each dilution gave identical results (i.e., all positive or all negative), the greatest dilution giving a positive result was considered to contain 1 PCR unit per replicate volume. When replicate assays were performed at a dilution such that not all of the replicates gave a positive reaction, the average amount of HCV RNA (in PCR units) per replicate was taken as −ln[fraction of negative reactions]. This method corrects for reactions that contain two or more template molecules, which cannot be distinguished from those containing only one molecule.[19]

Results

Quantitation of HCV RNA in plasma samples

We determined the sensitivity of the PCR assay for HCV RNA by performing limiting-dilution analysis on a sample of infectious plasma (H strain) known to contain 10^6 to 10^7 chimpanzee-infectious doses per mL.[24] In our assay, this sample contained 1.4×10^6 PCR units of HCV RNA per mL (Table 1). Thus, the PCR assay has a sensitivity comparable to the chimpanzee model. When plasma centers tested 3073 plasma donations from otherwise acceptable donors (i.e., who were nonreactive for antibody to human immunodeficiency virus type 1 and negative for hepatitis B surface antigen and who had alanine aminotransferase levels less than twice the upper limit of normal) for anti-HCV, 186 (6.1%) were repeatably reactive and 2887 were negative. A pool prepared from the anti-HCV-reactive donations contained 1.4×10^5 PCR units of HCV RNA per mL, whereas a pool prepared from the anti-HCV-negative donations contained 1.6×10^3 PCR units per mL (Table 1). From these data, it can be calculated that a pool comprising all 3073 units would contain $(186 \times [1.4 \times 10^5] + 2887 \times [1.6 \times 10^3])/3073 = 1.0 \times 10^4$ PCR units per mL. Thus, in this instance, anti-HCV screening decreased the viral load of the plasma pool by a factor of 6.

Partitioning of HCV RNA during fractionation of plasma to immune globulin

We subjected a portion of the anti-HCV-reactive plasma pool to cold ethanol fractionation (see Fig. 1) and tested the various fractions for HCV RNA by PCR. In the case of supernatants and suspensions, the RNA was extracted from 1 mL of solution. For analysis of solid fractions (i.e., cryoprecipi-

Table 1. *Limiting-dilution analysis of HCV RNA in plasma samples*

Sample* (vol)	Number positive/number tested at dilution of								PCR (U/mL) in sample†
	$10^{-2.5}$	$10^{-3.0}$	$10^{-3.5}$	$10^{-4.0}$	$10^{-4.5}$	$10^{-5.0}$	$10^{-5.5}$	$10^{-6.0}$	
Anti-HCV(+) pool (0.10 mL)				1/2‡	0/3	0/3			1.4×10^5
Anti-HCV(−) pool (1.0 mL)	1/1	2/4‡	1/4‡	0/2					1.6×10^3
H strain plasma§ (0.10 mL)						1/2‡	0/3	0/3	1.4×10^6

* RNA from the volume of plasma indicated was dissolved in 100 μL of water and serially diluted, and replicates (50 μL) were then subjected to RTR PCR.
† Calculated from the results observed at limiting dilutions.
‡ Limiting dilutions.
§ Plasma sample known to contain 10^6 to 10^7 chimpanzee-infectious doses of HCV per mL.

tate, fraction I, fraction II, and fraction III), one-fourth of the total precipitate obtained from the fractionation of 100 mL of plasma was suspended in 10 mL of phosphate-buffered saline, and the RNA was extracted from 1 mL of this solution. As shown in Fig. 2, when we tested undiluted RNA extracts of these samples, an amplified PCR product of the expected size (227 base pairs) was detected in all samples except supernatant II + IIIw, supernatant III, and supernatant II. The specificity of the RTR PCR was demonstrated with positive (H strain) and negative control plasmas tested concurrently. It is of interest that HCV RNA was detected in fraction II, but not in supernatant III (from which fraction II is prepared), which suggests that both of these materials contain low levels of HCV RNA.

To quantify further the HCV RNA content of these fractions, we serially diluted the RNA extracts from the samples prior to RTR and PCR. The results (Table 2) were expressed as total PCR units contained in the fraction, and, because these results were derived from 3.16-fold serial dilutions, their accuracy is probably limited to a factor of 3 or 4. Of the HCV RNA in the starting plasma, about 50 percent was brought down with cryoprecipitate. The value obtained for the HCV RNA content of fraction I (8×10^6 PCR units) appears to be high by a factor of 2 or 3, as this value plus that for supernatant I (3×10^6) should equal 6×10^6, the value for cryosupernatant. Thus, precipitation of fraction I removed about 50 percent of the HCV RNA remaining in cryosupernatant, so that supernatant I contained only 20 percent of the HCV RNA present in the starting plasma. Almost all of this was precipitated into fraction II + III; HCV RNA was detected in the supernatant II + III when tested without dilution, but not when tested at 1 in $10^{0.5}$ dilution. Suspension and reprecipitation of fraction II + III also resulted in the partitioning of all of the HCV RNA into fraction II + IIIw; none was detected in the supernatant II + IIIw. The same was true for the separation of fraction III, with virtually complete recovery of HCV RNA in this precipitate. Although HCV RNA was not detected in supernatant III, low concentrations were found in the fraction II derived from this supernatant and in the immune globulin produced from this fraction. Apparently, the level of HCV RNA in these materials was just at (or below) the limits of detectability (i.e., 1 PCR unit/sample volume). For increased accuracy, a solution of immune globulin containing 3.4-percent protein was prepared from the fraction II and examined for HCV RNA by limiting dilution. When 38-μL portions of this solution were subjected to RTR and PCR, 2 of 2 test samples yielded positive results; when 12-μL portions were used, only 3 of 10 samples were positive. Thus, the estimated concentration of HCV RNA in this sample was $-\ln(0.7)/0.012 = 30$ PCR units per mL. As this sample is enriched approximately five-fold in IgG over the starting plasma, and as the overall yield of IgG by the Cohn-Oncley process is about 50 percent, 30 PCR units per mL of immune globulin solution would correspond to about 3 PCR units per mL of starting plasma, which was found to contain 1.4×10^5 PCR units per mL. Thus, the overall clearance of HCV RNA achieved in preparing immune globulin by the Cohn-Oncley process was about 1.4×10^5 divided by 3 $= 4.7 \times 10^4$ (a 4.7 $\log_{10}$ reduction). Most of this reduction (4 $\log_{10}$) was the result of a single step (fraction III removal).

Table 2. *HCV RNA in plasma fractions from 100 mL of anti-HCV(+) pool*

Sample number	Description	Total HCV RNA (PCR U)	Total protein* (g)
	Plasma	1.4×10^7	6.00
1	Cryoprecipitate	6×10^6	0.33
2	Cryosupernatant	6×10^6	5.67
3	Fraction I	8×10^6	0.31
4	Supernatant I	(3×10^6)†	5.36
5	Supernatant II + III	3×10^2	4.06
6	Fraction II + III	(3×10^6)†	1.30
7	Supernatant II + IIIw	$<2 \times 10^2$	0.07
8	Fraction II + IIIw	3×10^6	1.23
9	Fraction III	3×10^6	0.70
10	Supernatant III	$<2 \times 10^2$	0.53
11	Fraction II	1×10^2	0.54
12	Supernatant II	$<2 \times 10^2$	0.00
13	Immune globulin	3×10^2	0.41

* Calculated from the net weight of the precipitates, assuming each to contain 25 percent protein. The starting plasma was assumed to contain 6 percent protein.
† These samples were not titrated, but were positive when tested undiluted. The numbers in parentheses are minimum values, based on the results for fraction II + IIIw and fraction III.

Discussion

It has been estimated that anti-HCV testing of plasma will detect about 80 percent of infectious donations.[6] However, from this it cannot be assumed a priori that screening plasma for anti-HCV will result in an 80-percent decrease in the concentration of HCV in plasma pooled from thousands of donations. The effectiveness of anti-HCV screening in reducing the viral load of plasma pools depends not only upon the efficiency with which the test detects infectious units, but also upon the relative viral titers in seropositive units and false-negative units, that is, those units containing HCV but devoid of anti-HCV.[7] If, on average, the latter contain HCV at substantially higher levels than the former, then anti-HCV screening will not be as effective in reducing the viral load of the pool as it is in reducing the number of infectious units pooled. The work reported here demonstrates that anti-HCV screening reduces the concentration of HCV in a large plasma pool by a factor of about 6 (83%), which is in good agreement with the reported sensitivity of the test. Thus, it appears that there is not a great disparity in the average viral titers of anti-HCV-reactive units and false-negative units.

During Cohn-Oncley fractionation, most of the HCV RNA partitions into cryoprecipitate, fraction I, and fraction III, with trace amounts also found in supernatant II + III and fraction II. Plasma derivatives produced from cryoprecipitate (anti-hemophilic factor), cryosupernatant (factor IX complex), and fraction I (fibrinogen, no longer licensed in the United States) are known to transmit HCV unless additional virus-inactivation steps are incorporated in their manufacture, so the observation that these fractions contain HCV RNA is not surprising. Products derived by further fractionation of supernatant II + III include antithrombin III, alpha-1 proteinase inhibitor, plasma protein fraction, and albumin, all of which are subjected to heating for 10 hours at 60°C. The fact that these products do not transmit HCV may be due more

to virus inactivation by the heating step than to physical removal of virus by the fractionation process. Because of the low level of HCV RNA found in supernatant II + III (3 PCR units/mL), it was not possible to follow the distribution of viral RNA through the final fractionation steps leading to albumin.

The detection of HCV RNA in fraction II, albeit at low levels, is noteworthy. Our work used a plasma pool "enriched" in HCV by a factor of 15 over an unscreened pool and by a factor of 90 over an anti-HCV-screened pool. Nevertheless, if the efficiency of virus removal is independent of the viral load in the starting plasma, one might expect to find substantial amounts of HCV RNA in immune globulin prepared from unscreened pools or even in that from anti-HCV-screened pools (60 and 10 PCR units of HCV RNA/g IgG, respectively). Given that therapeutic doses of immune globulin range from 0.003 to 2 g per kg of body mass, and given the approximate equivalence of our PCR unit to a chimpanzee-infectious dose of HCV, it might seem that this product would have a high likelihood of transmitting HCV. There are several possible reasons why it apparently does not do so. 1) Intravenous immune globulin products undergo additional manufacturing steps that may, fortuitously, have virucidal properties; 2) the HCV RNA in immune globulin may not be infectious because of possible disruption of the viral envelope during the fractionation process; and 3) antibodies may be present in the immune globulin fraction that neutralize the infectivity of HCV. In spite of an excellent safety record, the detection of HCV RNA in immune globulin, coupled with sporadic reports of non-A,non-B hepatitis transmission by intravenous immune globulin, suggests that virus-inactivation and/or -removal steps should be considered for immune globulin products.

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