Prevalence of Hepatitis C Virus in Plasma Pools and the Effectiveness of Cold Ethanol Fractionation

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ABSTRACT

Screening blood donations for antibodies against hepatitis C virus (HCV) greatly reduces the risk of transmitting HCV by transfusions. However, despite such screening programs, plasma pools still contain a high percentage of HCV ribonucleic acid as determined by polymerase chain reaction. This result would not be alarming if the procedures for producing blood products included steps to inactivate or remove HCV. Although this appeared to be the case for all blood products, such as coagulation factors and most immunoglobulins, which are subjected to an inactivation step, the effectiveness of the cold ethanol fractionation process still needed to be determined. In validation experiments using bovine viral diarrhea virus as a model virus for HCV, we demonstrated that the Cohn-Oncley cold ethanol fractionation process neither inactivated nor removed this virus sufficiently.

Our observations may help to explain how HCV was transmitted to a number of recipients of intravenous immunoglobulin.

INTRODUCTION

Hepatitis C virus (HCV) causes most cases of transfusion-associated non-A, non-B hepatitis (NANB). Based on identification of the HCV genome in 1989, diagnostic tools were developed to detect antibodies directed against HCV. The introduction of these diagnostic tools into the screening program for blood donations significantly reduced the incidence of transfusion-associated NANB.

From their experience with transfusion-associated NANB, investigators concluded that screening plasma used to manufacture blood products would increase the margin of safety, even though blood products were regarded as safe with respect to HCV transmission at that time (after 1990). The...
safety of these products was attributed mainly to the virus inactivation steps introduced into the production procedures for coagulation factors since the recognition of human immunodeficiency virus transmission in the early 1980s. Also, immunoglobulins appeared not to be involved in HCV transmission, even though some of the licensed products were not subjected to a deliberate virus inactivation step and certain lots of intravenous immunoglobulins (IGIV) appeared to have infected a number of recipients. In this ambiguous situation, doubts were raised about the usefulness of anti-HCV screening of plasma donations used to manufacture blood products, especially IGIV. In addition, an increase in the risk of HCV transmission by IGIV was not ruled out. Nevertheless, anti-HCV screening of source plasma was introduced under the impression that detection of anti-HCV antibodies often correlates with infectivity in the same serum or plasma. In the European Union, only lots of blood products produced from donations testing negative in an anti-HCV antibody assay have been released to the market since January 1, 1993. We were interested in studying the influence of this screening program on the HCV load in plasma pools and in investigating the effectiveness of the Cohn-Oncley cold ethanol fractionation process in inactivating or eliminating HCV.

MATERIALS AND METHODS

Plasma pools and immunoglobulins were obtained from different suppliers of blood products in Germany (Armour, Eschwege; Baxter, Munich; Behringwerke, Marburg; Biotest, Dreieich; DRK Hagen, Hagen; Sandoz, Nuremberg; and Tropon, Cologne). The different suppliers were coded with the letters A to G. Information concerning the geographic origin of individual donations was available for some of the plasma pools.

Polymerase Chain Reaction Testing for Hepatitis C Virus Ribonucleic Acid

The presence of HCV ribonucleic acid (RNA) in plasma pools or final products was investigated by using either the Amplicor® HCV polymerase chain reaction (PCR) kit (Hoffmann-La Roche, Nutley, New Jersey) (100-µL sample volume) or, after reverse transcription (RT), by an in-house nested PCR with the 5'-noncoding region as the target (200-µL sample volume). Both methods were applied without prior ultracentrifugation.

The in-house nested RT-PCR was roughly quantitated by scoring whether HCV-specific bands stained with ethidium bromide were already detectable in agarose gels after the first PCR run or only after the second run. For the Amplicor HCV PCR, serial dilutions in HCV-negative plasma were performed before RNA extraction to obtain a semiquantitative estimation.

Viruses and Cells

The Osloss strain of bovine viral diarrhea virus (BVDV), a pestivirus (family Flaviviridae), was provided by J. Thiel (Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen, Germany). Semliki Forest virus (SFV), a togavirus (family Togaviridae), was obtained from G. Pauli (Robert-Koch-Institut, Berlin, Germany). BVDV was cultured on Madin-Darby bovine kidney (MDBK) cells (American Type Culture Collection [ATCC] CCL-22) and SFV on Vero cells.
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(ATCC CCL-81). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. SFV stock suspensions were harvested from the culture supernatant in Vero cells. BVDV stocks were made from infected MDBK cell lysates.

Cohn-Oncley Fractionation

The Cohn-Oncley fractionation procedure used in the virus validation studies is summarized in Figure 1. It follows Cohn method $^{6,16}$ and Oncley method $^{9,17}$. Virus-spiking experiments were assessed for each precipitation step. Ethanol concentrations (v/v), pH values, and temperature were adjusted as indicated in Figure 1. Frozen plasma stored at -70 °C was thawed slowly at +4 °C and the cryoprecipitate was removed by centrifugation at 10,000 × g for 15 minutes at +4 °C. The 300 mL of cryo-poor plasma was spiked with 33 mL of virus stock suspension and subjected to fractionation. The addition of virus was repeated for each step except for supernatant I.

After mixing, three 1-mL samples were taken to determine the initial virus load. The material was cooled to the indicated temperature, and the pH was adjusted by using a pH 4.0 buffer (0.4-M acetic acid, 0.08-M sodium acetate). Ethanol was added while stirring to achieve the calculated concentration within 45 minutes. After completing the ethanol addition, the pH was readjusted using adequate amounts of an acetate buffer (2.0-M sodium acetate or pH 4.0 buffer) to maintain the ethanol concentration. The suspensions were stirred for the time indicated in Figure 1. Three samples of 1 mL each were drawn before separation of the precipitates by centrifugation at -5 °C and 10,000 × g for 15 minutes. The supernatants were collected. The precipitates were homogeneously suspended at +4 °C in distilled water (0.45% sodium chloride for fraction II).

Three 1-mL samples were taken from the supernatant and from the suspended precipitate. Precipitate I, supernatant II/III, precipitate III, and supernatant II were discarded. Supernatant I, precipitate II/III, precipitate II/III W, supernatant III, and fraction II were used to prepare immunoglobulin. Precipitate II/III was suspended in 12 volumes of ice-cold distilled water to which 112 μL of 0.5-M disodium phosphate (pH 9.2) had been added, precipitate II/III W was suspended in 17 volumes of acetate buffer, and precipitate II was resuspended in 10 volumes of 0.45% sodium chloride solution. A 1-mL sample from each fraction was titrated immediately after drawing and two aliquots of 1 mL each were stored at -70 °C. Additionally, an aliquot from the virus stock was titrated as a positive control.

Determination of Virus Titers

Virus titers were quantitated by tissue culture infectious dose (TCID) assays. MDBK or Vero cells (for SFV and BVDV, respectively) were grown to confluence in 96-well microtiter plates. The sample inoculum was 0.05 mL per well. Serial threefold dilutions were made in DMEM without serum, with eight replicates per dilution step. One row of wells on the plates was designated as negative control. The virus material was allowed to adsorb onto the cells for 60 minutes at 37 °C for SFV and for 90 minutes at 37 °C for BVDV. The 100-μL DMEM supplemented with 15% fetal calf serum was then added to each well. The plates were
Figure 1. Description of the procedure and parameters of the Cohn-Oncley cold ethanol fractionation process used in the virus validation study. Susp. = suspension; Sup. = supernatant; Ppt. = precipitate.
Table I. Results of hepatitis C virus (HCV) ribonucleic acid (RNA) testing by polymerase chain reaction (PCR) in plasma pools collected by supplier A before and after introduction of anti-HCV screening.

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<tbody>
<tr>
<td>8</td>
<td>Not screened</td>
<td>8</td>
<td>8 (100%)</td>
<td>8 (100%)</td>
</tr>
<tr>
<td>24</td>
<td>Screened</td>
<td>0</td>
<td>0 (0%)</td>
<td>2 (8.3%)</td>
</tr>
</tbody>
</table>

*Testing by using in-house nested PCR.

incubated at 37 °C (2 days for SFV and 3 days for BVDV). Cytopathic effects of SFV and BVDV were checked by microscopic examinations. Infectivity at 50% (TCID\textsubscript{50}) was calculated according to Karber.\textsuperscript{18} The virus load was calculated from virus titers and volume of test material and is expressed in terms of log\textsubscript{10}. The reduction factors also are expressed in terms of log\textsubscript{10}.

RESULTS

In a pilot study, we investigated whether excluding plasma donations that were reactive in an anti-HCV antibody assay had any effect on the HCV load of plasma pools (Table I). In samples of eight plasma pools from unscreened donations, not only antibodies but also HCV RNA could easily be detected in every case. However, because of the small number of samples, it is difficult to estimate the overall percentage of plasma pools that might have been contaminated. In plasma pools from screened donations, HCV could not be detected initially. However, in a further set of experiments, two pools were found in which a signal appeared in the second run of the nested PCR. From these data, we concluded that excluding reactive donations clearly contributes to reducing the virus load in plasma pools but does not ensure a complete disappearance of HCV.

We also tested a limited number of immunoglobulin preparations produced from some of these plasma pools (two from unscreened donations and one from screened donations). In no case could HCV RNA be detected by PCR. This was also true for some other commercially available IGIV lots.

In early 1994, evidence emerged that a number of lots of the IGIV preparation Gammagard\textsuperscript{®*} were involved in the transmission of HCV. A small number of Gammagard lots were subsequently tested using PCR.\textsuperscript{14} In contrast to earlier findings, five of these lots were found to be HCV RNA positive. From other studies,\textsuperscript{19} it became clear that a substantial number of IGIV lots were indeed contaminated with HCV. Obviously, this was at odds with the prevalence of HCV in plasma pools as predicted by our pilot study. For this rea-

\textsuperscript{*}Trademark: Baxter Healthcare Corporation, Hyland Division, Glendale, California.
Table II. Results of hepatitis C virus (HCV) ribonucleic acid (RNA) testing\(^1\) by polymerase chain reaction (PCR) in samples of plasma pools from anti-HCV-screened donations provided by suppliers B through F.

<table>
<thead>
<tr>
<th>Supplier</th>
<th>No. of Plasma Pools Tested</th>
<th>No. of Plasma Pools HCV RNA–Positive</th>
<th>No. of Plasma Pools HCV RNA–Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>177</td>
<td>72 (41%)</td>
<td>105 (59%)</td>
</tr>
<tr>
<td>C</td>
<td>28</td>
<td>10 (36%)</td>
<td>18 (64%)</td>
</tr>
<tr>
<td>D</td>
<td>44</td>
<td>0 (0%)</td>
<td>44 (100%)</td>
</tr>
<tr>
<td>E</td>
<td>14</td>
<td>3 (21%)</td>
<td>11 (79%)</td>
</tr>
<tr>
<td>F</td>
<td>21</td>
<td>3 (14%)</td>
<td>18 (86%)</td>
</tr>
</tbody>
</table>

\(^1\)Testing by using Amplicor\(^\circledR\) HCV PCR kit.

Contrary to what was generally expected, these studies showed that despite the screening of donations using anti-HCV assays, plasma pools can still be contaminated with HCV. The percentage of positive pools varies from supplier to supplier, but appears to be highest when donations from the United States are included. These findings also demonstrated that one condition for HCV transmission by IGIV preparations still remains, namely, the presence of HCV in the starting material (the plasma pools).

Even in this context, the risk of transmitting HCV could be greatly reduced (if

Table III. Results of hepatitis C virus (HCV) ribonucleic acid (RNA) testing\(^4\) by polymerase chain reaction (PCR) in plasma pools collected by supplier G from two different geographic areas.

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<tbody>
<tr>
<td>111</td>
<td>United States</td>
<td>57 (51%)</td>
<td>54 (49%)</td>
</tr>
<tr>
<td>59</td>
<td>Europe</td>
<td>11 (19%)</td>
<td>48 (81%)</td>
</tr>
</tbody>
</table>

\(^4\)Testing by using Amplicor\(^\circledR\) HCV PCR kit.
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not eliminated) if HCV were efficiently inactivated or removed during IGIV production. Therefore, we need to know whether Cohn-Oncley cold ethanol fractionation contributes to virus inactivation or removal. However, studies are hindered because HCV cannot be cultured in tissue culture and, therefore, cannot be quantified by titration. Detection of HCV is restricted to nucleic acid amplification methods that cannot be easily used for accurate quantitation. An example of a study using PCR is given by Yu et al.20

Another possibility is the use of indicator viruses that may mimic the behavior of HCV during a given process. Because pestiviruses appear to be the closest relatives to HCV with regard to genome organization and physical characteristics (ie, size and buoyant density),21-23 the pestivirus BVDV was chosen for virus validation studies. In addition, we also investigated the behavior of the togavirus SFV, which has also been proposed as a model virus for HCV.

The Cohn-Oncley fractionation process consists of a series of ethanol precipitation steps. The variables are ethanol concentration, pH, temperature, and incubation time (Figure 1). Two principal effects on viruses are possible—inactivation by ethanol or partitioning between precipitate and supernatant. Inactivation is measured by comparing the virus load in the spiked starting material with the virus load in the suspension obtained after the addition of ethanol (according to the conditions specific to each step). The effects of partitioning are reported as differences in virus load in the supernatant and precipitate (Figure 2). We repeated the experiments three times, with practically the same results. The data described here represent a single experimental run.

If the virus load in the spiked starting material (designated A in Figure 2) is compared with the virus load in the respective suspension (B), no reduction can be observed. This is true for ethanol concentrations up to 25% (step 5) and for both viruses tested.

Analysis of the behavior of viruses in partitioning should first focus on SFV. The data clearly showed a concentration of virus in the precipitates (compare D with A or B for every step). With the exception of step 1, the virus load in the supernatant is reduced by approximately four log steps, that is, by a factor of 10,000 (compare C with A or B). However, in the production of immunoglobulins, the precipitates are usually further processed (precipitates of steps 2, 3, and 5). As the virus load in the supernatant of step 1 is only slightly reduced, only step 4 of the Cohn-Oncley process contributes significantly to reducing SFV during the production of immunoglobulins.

For BVDV, similar, but not identical, behavior was observed. Again, the virus was found mainly in the precipitates and to a lesser degree (with the exception of step 1) in the supernatant. However, the reduction was less pronounced, usually about three log steps. For step 4, a marked difference between SFV and BVDV was observed. Although the SFV load in the supernatant (which is further processed) was reduced by 4.6 log steps, the BVDV load was decreased by a factor of only 40 (1.6 log steps).

DISCUSSION AND CONCLUSION

In 1993 and 1994, a commercial IGIV preparation was involved in the transmission of HCV to a considerable number of recipients (see Gomperts24). Our data demonstrated the existence of two essential prerequisites for this incident: (1) a high
Figure 2. Effectiveness of Cohn-Oncley fractionation in virus reduction ($\log_{10}$) for Semliki Forest virus (SFV) (a togavirus) and bovine viral diarrhea virus (BVDV) (a pestivirus). Infectivity values in the different fractions and for the different steps of the fractionation are expressed as virus load $\log_{10}$ TCID$_{50}$. TCID = tissue culture infectious dose; Susp. = suspension; Sup. = supernatant; Ppt. = precipitate.

prevalence of HCV in plasma pools originating from US donations, despite screening with second-generation anti-HCV antibody testing; and (2) the limited capacity of the Cohn-Oncley fractionation process to inactivate or remove pestiviruses.

For many diseases caused by viruses (e.g., poliomyelitis, hepatitis A, and measles), the presence of antibodies indicates that the respective viruses have been cleared from the body. This is definitely not true for the human immunodeficiency virus. For successful replication, this retrovirus must integrate its own genome into the DNA of the host cell and therefore persists lifelong, even in the presence of an-
antibodies. From clinical observations, researchers concluded that HCV persists in most cases, although the molecular mechanism is still unknown. In such situations, the presence of antibodies may also indicate the presence of infectious viruses, at least in cases of chronic infection. Indeed, excluding donations that are reactive with the first generation of assays that detected antibodies against a nonstructural HCV protein reduced the risk of HCV transmission by blood donation. The introduction of second-generation assays that recognized antibodies against a structural protein reduced this risk to almost zero. The virus load in plasma pools was expected to be considerably reduced after screening of all single donations. This was suggested by the results of our pilot study. Surprisingly, however, a more extended study revealed that a high percentage of plasma pools are still contaminated with HCV. Yet these results can be considered somewhat uncertain, because PCR techniques are associated with a high number of false-positives. However, data obtained by two different collaborative studies for the performance of HCV PCR (the second Eurohep study and the study performed by the National Institute for Biological Standards and Control in the United Kingdom, J. Saldanha and P. Minor, unpublished data) established that the number of HCV RNA-positive plasma pools is underestimated rather than overestimated. In addition, 30 coded samples were submitted by one supplier to another laboratory, which reported results virtually the same as ours (28 samples with identical data and 2 with divergent data [1 positive and 1 negative in each of both laboratories]). Another argument in favor of our data is that several final lots of IGIV, rather than a single one, were involved in the most recent HCV transmission event. This means that a number of plasma pools from which IGIV was produced must have been contaminated.

There are two possible explanations for the high percentage of HCV RNA positivity in plasma pools: (1) a high incidence of HCV infections combined with a long window of infectivity period and a high virus titer during that time; and (2) the existence of antibody-negative virus carriers. The long window period and high virus titer early in HCV infection have already been described by Simmonds. The incidence of HCV infections in blood and plasma donors may vary and may explain the discrepancies in the percentage of HCV contamination in plasma pools of European or American origin. The reason for this deserves further investigation. The possibility of antibody-negative virus carriers should not be ruled out. In immunocompromised individuals, HCV may persist without the induction of a humoral immune response. In our own studies of a cohort of 252 persons infected with human immunodeficiency virus (from risk groups of intravenous drug users and male homosexuals) without antibodies against HCV (M. Nübling et al., unpublished data, 1995), 5 were found to be positive for HCV RNA by PCR. Such studies are difficult to perform in otherwise healthy individuals. However, Thomssen et al. observed that HCV binds to low-density lipoproteins, which could lead to masking of the virus and prevent the induction of antibodies.

The presence of HCV in plasma pools would not be disastrous if the virus were inactivated or removed during the subsequent purification steps. Usually, the first steps consist of an ethanol fractionation according to either Cohn et al. and On-
Early virus validation studies using PCR technology to detect HCV RNA showed a reduction of the virus load using these purification steps, but virus RNA could still be detected in fraction II, the raw immunoglobulin (precipitate II in Figure 1). We chose a different approach for our validation studies because it is difficult to accurately quantitate the titer of infectious viruses by nucleic acid amplification methods and because HCV cannot be grown in tissue culture, and, therefore, cannot be titrated by classic virologic means. According to the EEC Note for Guidance, Validation of Virus Removal and Inactivation Procedures, we selected two model viruses for HCV: SFV, a togavirus similar to HCV in genome size and overall structure (RNA genome, capsid, and lipid envelope), and BVDV, a pestivirus. Some data (genome organization and buoyant density) support the suggestion that HCV is most closely related to pestiviruses even though it forms a separate genus in the family of Flaviviridae.

Our virus validation data clearly demonstrated that, despite ethanol concentrations up to 25%, the cold ethanol fractionation process had no inactivating effect on either virus studied. Other viruses investigated in our laboratory (eg, duck influenza virus and poliovirus) also were not inactivated. These results are in agreement with some published data.

The effect of cold ethanol fractionation on the virus load broadly amounts to partitioning the viruses into precipitates and removing these precipitates. An exception is step 1, in which only a low ethanol concentration is used and the virus distributes equally into supernatant and precipitate. Intermediate materials in the production of immunoglobulins are the supernatants after steps 1 and 4 and the precipitates after steps 2, 3, and 5. Thus only step 4 contributes significantly to reducing the virus load, usually by a factor of 10,000 or more. This is true for most viruses studied in our laboratory. The only exceptions are pestiviruses. These viruses are enriched in the precipitate of step 4, but usually by a factor of only 40 to 100. The most obvious difference between step 4 and all the other steps is the low pH (5.4 as opposed to 6.8 to 7.2). However, whether the low pH influences the behavior of pestivirus during ethanol fractionation is unclear.

A factor that considerably differentiates BVDV from most other viruses is its low buoyant density, which is about 1.1 g/mL. HCV also has a buoyant density of less than 1.1 g/mL, at least in plasma. The suspension in step 4 may have a higher density so that both viruses float in the solution rather than precipitate. If so, whether the precipitate is separated by centrifugation or filtration could be important. Floating viruses may be trapped in the filter cake but decanted along with the supernatant after centrifugation. This effect still must be investigated.

The low density of HCV results from binding to low-density lipoproteins and nonspecific interaction with other plasma components. However, HCV can also be found in antigen-antibody complexes that possess a higher density (1.2 g/mL). Such complexes may behave differently during the fractionation process. Therefore, BVDV as a model virus would reflect a situation in which antibodies are lacking, because human plasma probably does not contain antibodies against a cattle virus. Our validation data could support the hypothesis of Yu et al. that a lack of antibodies may increase the risk of HCV...
transmission by immunoglobulin preparations whose production is based solely on a cold ethanol fractionation process.

ACKNOWLEDGMENTS

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REFERENCES


21. Miller RH, Purcell RH. Hepatitis C virus shares amino acid similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. Proc Natl Acad Sci USA. 1990;87:2057–2061.


