CONCISE COMMUNICATION

Hepatitis A Virus Infection in Tamarins: Experimental Transmission via Contaminated Factor VIII Concentrates

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An experimental hepatitis A virus (HAV) transmission study was performed in 3 tamarins, using a factor VIII concentrate linked to a recent outbreak of HAV infections in German hemophiliacs. The typical indicators for HAV infection were investigated in feces and serum samples. One tamarin showed a classical HAV infection with seroconversion. HAV antigen and HAV RNA were detected in feces of a second animal, but no seroconversion was observed until 19 weeks after inoculation. The HAV sequences from the reverse-transcription– and polymerase chain reaction–positive samples of the 2 animals were identical to the deduced HAV sequences of the chain of infection (from plasma pool to final product to patients). The study results provide conclusive evidence of the presence of infectious HAV in coagulation factor concentrate. Because a number of HAV transmission episodes have been described for solely solvent/detergent-treated factor VIII preparations, continued use of these agents seems questionable.

Hepatitis A virus (HAV), a member of the picornavirus family, is usually transmitted by the fecal-oral route. However, hepatitis A viremia may result in a parenteral transmission of this virus. HAV is both highly stable and infectious, and current plasma fractionation and solvent/detergent (S/D) inactivation procedures may not totally remove or inactivate residual virus from contaminated blood preparations. A new outbreak of hepatitis A infection in hemophiliacs treated with the same lot of S/D-treated factor VIII preparation occurred in Germany in 1997. This transmission could be traced to the inclusion of a viremic plasma unit in the starting material. The chain of infection from plasma pool to final product to patients was shown by molecular approaches [1].

Replication-competent infectious virus can be assessed in animal models by use of chimpanzees, owl monkeys, or tamarins or marmosets [2]. There are no comparably sensitive cell culture techniques, and there have been no reports of successful in vivo infection experiments with polymerase chain reaction (PCR)–positive factor VIII concentrates [3, 4], perhaps because of the very low virus load in the factor VIII lots, which could result in an uneven virus distribution in individual vials of the final product. An additional explanation might be the great discrepancy between the PCR-calculated virus titer and the titer measured by infectivity assays. Furthermore, the inoculum in unsuccessful experiments might not have contained sufficient infectious HAV particles. It appears to be difficult to design an animal transmission study, since large amounts of factor VIII preparation must be administered. Nevertheless, the manufacturer of the agent linked to an HAV outbreak among German hemophiliacs initiated animal studies with the implicated factor VIII lot. Susceptible New World primates of the Saguinus species were used for the experimental infection.

Materials and Methods

Animals

Three adult saddleback tamarins (Saguinus fuscicollis) were caged individually but in acoustic contact with each other. Special care was taken to minimize smear infection, fecal dust, and aerosols. Preinoculation sera were collected at days 65, 57, 43, 30, and 9 before the start of the study. After inoculation, the animals were examined clinically and bled weekly. Bleeding and inoculation were done under ketamine anesthesia. Fecal samples were collected each day. All specimens were frozen and stored at −70°C.

Preparation of Inocula

For the studies, we used 84 vials of the implicated factor VIII lot (each 1000 U of factor VIII in 10 mL after reconstitution). HAV was concentrated by ultracentrifugation over 30% (wt/wt) sucrose cushion.
ion in TNA buffer (10 mM Tris [pH 7.4], 100 mM NaCl, and 1 mM EDTA). Pelleted material was resuspended carefully in 0.9% NaCl and was kept at 4°C.

**Inoculation**

Each animal was inoculated intravenously with 1 mL of the re-suspended material (corresponding to an equivalent of 28,000 factor VIII U of the implicated lot). The animals were observed until post-inoculation (pi) day 132.

**Indicators of Infection**

Alanine aminotransferase (ALT). The enzyme activity was measured by an autoanalyzer (Roche/Hitachi Modular Analytics, Roche Diagnostics). The assay was performed with 200 μL of serum. HAV antibodies (anti-HAV). The presence of anti-HAV was determined by commercial ELISA (Enzygnost anti-HAV and anti-HAV IgM; Dade Behring).

**HAV antigen.** Fecal suspensions were prepared at a 10% (wt/vol) concentration in PBS (pH 7.4) and were clarified at 2000 g for 10 min at 4°C. A 100-μL aliquot of the supernatant was used for the HAV antigen EIA (Mediagnost), according to the manufacturer’s instructions.

**HAV RNA.** As was done previously, the presence of viral nucleic acid was determined in supernatants of fecal suspensions and in serum specimens by reverse-transcription (RT) and nested PCR amplification, using primers targeted to the C-terminus of the VP3 region and to the VP1/P2A junction [1]. Procedures designed to avoid contamination were strictly applied throughout the studies (strict spatial separation of reagent preparation, sample preparation, amplification, and detection). Controls were implemented to monitor the PCR assay. The diagnostic laboratory is accredited as having the competence to perform all investigations under the European standard EN 45001.

**HAV RNA Sequencing**

The HAV region spanning the VP1/P2A junction provides a tool for distinguishing one strain from another. For DNA sequencing, an aliquot of the amplified PCR product was directly cloned into the pCR2.1 vector with the TA cloning system, according to the manufacturer’s instructions (Invitrogen). Plasmid DNA, with an insert of the expected size, was sequenced with the HAV-specific primer (inner sense) by using the 373 DNA sequencer stretch line (PE Applied Biosystems).

**Phylogenetic Analysis**

Nucleotide sequences were aligned with the CLUSTAL W program (http://genome.dkfz-heidelberg.de). The phylogenetic tree was constructed from the distance matrix by the unweighted pairwise group method of arithmetic averages in the neighbor program with a phylogenetic software package (PHYLIP version 3.5c; University of Washington) [5].

**Results**

The study was designed for 3 tamarins (S. fuscicollis) known to be susceptible to HAV infection. In phase I of this study, each animal was inoculated with the same volume of the incriminated factor VIII material. If none of the animals had developed any sign of HAV infection, the monkeys would have been reinoculated with an HAV-negative factor VIII preparation spiked with HAV strain GR08 at a concentration known to infect tamarins (phase II) [4]. Possible sources for HAV infection of these animals other than the experimental virus transmission could be ruled out. In the animal laboratory facilities, no HAV was handled before this study, and laboratory personnel were vaccinated against HAV. The results of investigations with the collected preinoculation sera of the 3 animals showed that the tamarins were seronegative for HAV and that ALT levels were not elevated.

The inoculum volume was limited, due to the small size of the test animals. The crucial point was to place sufficient HAV particles into this inoculum. HAV in the factor VIII preparation had to be concentrated without appreciable loss of infectivity. HAV can easily be concentrated by ultracentrifugation because of the buoyant density of the virus particle, but this entails a considerable loss of infectivity due to the enormous centrifugal forces. Centrifugation through a sucrose cushion could reduce this problem. Examination of the pellets and the supernatants by RT-PCR showed that HAV had been concentrated.

Each animal received an equivalent of 28,000 U of the implicated lot of factor VIII in a 1-mL volume by intravenous injection without adjuvants. Animal 1 died at pi day 30 without signs of HAV infection. Figure 1 summarizes results for animals 2 and 3. As the first indicator of HAV infection, HAV RNA was detected in serum of animal 2 at pi day 14. Eleven days later, feces were HAV RNA positive, and, 2 additional days later, virus antigen was detected in fecal samples. ALT was significantly elevated by pi week 4 and peaked at pi day 35. Significantly increased ALT levels were also observed between pi days 82 and 90. This was traced to heavy intramuscular and subcutaneous treatment of a stress-induced diarrhea of animal 2 at pi days 80–82 (data not shown). At about pi week 6, anti-HAV IgM and anti-HAV IgG/IgM were detected. A titer of anti-HAV IgG could still be determined during the study period.

Animal 3 showed HAV RNA in fecal samples at pi days 25–38. Later samples were not investigated. During this period, viral shedding was also noted by the detection of trace amounts of HAV antigen. ALT levels were slightly elevated during pi days 7–14, 28–42, and 70. No seroconversion was observed during the study period. HAV sequences of the RNA-positive materials (serum and fecal specimens of tamarin 2 and fecal specimens of tamarin 3) were analyzed over the VP1/P2A junction (286 nt). The sequences from all samples were identical. There was 100% identity between these sequences and those amplified from the implicated factor VIII lot, the plasma pool, and the recipients (patients 1–4, 6, and 7).
Discussion

Despite the implementation of virus inactivation or elimination procedures in the manufacture of blood products, virus transmissions still occur. Although the S/D treatment was a major step toward inactivation of enveloped viruses, including hepatitis B and C viruses and human immunodeficiency virus, it does not affect nonenveloped viruses such as HAV and human parvovirus B19. The results of the molecular approaches revealed that the new cluster of hepatitis A disease among hemophiliacs in Germany was linked to factor VIII concentrates treated for virus inactivation solely by the S/D procedure. In addition, sera from the anti-HAV-negative patients (patients 5–7) treated with the incriminated factor VIII lot were included in the molecular analysis (table 1). These samples were not available at the time of the first study [1].

No virus-specific RNA was detected in patient 5. The period between the onset of hepatitis symptoms and the date of serum collection was longer than for the other patients. The findings obtained with the sera of patients 6 and 7 confirm the results of the previous study [1]. Of note, only patients 1–6 developed acute hepatitis A. Patient 7 was also infected with HAV but with no signs of acute illness. Although this patient was HAV vaccinated 56 days after the first infusion of the implicated factor VIII lot, HAV RNA was detected in a plasma sample collected at a late bleeding date (table 1). This indicates a longer period of viremia than usually considered.

The results of a prolonged duration of HAV viremia are supported by the findings of Yotsuyanagi et al. [6] and Bower et al. [7]. Moreover, there is an indication of HAV infection transmission despite vaccination [8]. Molecular approaches, as described here, are useful for tracing the chain of virus transmission. In con-

Table 1. Antibody status of hepatitis A virus (HAV) antibody–negative patients treated with factor VIII lot before risk factors for HAV infection were known.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at time of beginning infusion, years</th>
<th>Total amount of factor VIII administered, U</th>
<th>Days from first infusion to</th>
<th>HAV RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>First detection of symptoms</td>
<td>First detection of anti-HAV IgM</td>
</tr>
<tr>
<td>1</td>
<td>44</td>
<td>12,000</td>
<td>49</td>
<td>51</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>60,000</td>
<td>49</td>
<td>54</td>
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<td>3</td>
<td>60</td>
<td>44,000</td>
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<td>120,000</td>
<td>52</td>
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<td>25</td>
<td>60,000</td>
<td>40</td>
<td>47</td>
</tr>
<tr>
<td>6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21</td>
<td>4000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14</td>
<td>109&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>43</td>
<td>17,000</td>
<td>None</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOTE. ND, not determined; RT-PCR, reverse-transcriptase polymerase chain reaction.

<sup>a</sup>Patient with von Willebrand disease.

<sup>b</sup>Bolus infusion.

<sup>c</sup>At onset of symptoms, anti-HAV IgM was not determined.

<sup>d</sup>Patient received HAV vaccination 56 days after first factor VIII infusion.
trag, animal models for infectivity studies were not successful earlier [3, 4]. Nevertheless, the manufacturer again initiated animal studies, although the number of animals was limited to 3 tamarins.

There is no exact knowledge about the minimal infectious dose of HAV for humans and animals. In the recent factor VIII transmission episode, patient 6 received a bolus infusion of 4000 U of factor VIII (corresponding to a volume of 40 mL) of the contaminated lot and developed hepatitis A. This corresponds to about 10^4 HAV particles, as shown by molecular study results [1]. Because the calculation of virus load was based on statistical analysis and a considerable loss of HAV by concentration techniques was expected, the inoculum for the animal study was to correspond to \( \approx 28,000 \) factor VIII U in a 1-mL volume.

Although 1 of the 3 tamarins died on pi day 30 without signs of hepatitis A, animal 2 developed a classical HAV infection that included seroconversion. All nonspecific and specific indicators of HAV infection were detected. However, tamarin 3 excreted the virus, but no viremia was observed. In an earlier study, tamarins were experimentally infected with HAV RNA–positive human stool specimens, but no sign of viral infection was detected [9]. It is possible that tamarins are not the ideal host for human HAV strains [10]. In general, HAV disease in nonhuman primates is usually milder after experimental parenteral and oral infection, but the course of infection is similar to that in humans [11].

We believe that our study results are the first to show that the presence of infectious HAV in a coagulation factor concentrate can be demonstrated by experimental infection of HAV-susceptible animals (S. fuscicollis). HAV infection due to contamination by sources other than the experimental inoculation could be ruled out. Special care was taken before and during the study to prevent HAV contamination. The determined HAV sequences (VP1/P2A junction) from the RT-PCR–positive samples (sera and feces) of animals 2 and 3 were identical to the deduced HAV sequences of the chain of infection from plasma pool to final product to patients.

All data from in vitro and in vivo experiments give conclusive evidence that hepatitis A was transmitted by S/D–treated factor VIII concentrate. The transmissions were due to the inclusion of a viremic plasma unit in the starting material. The use of highly purified factor VIII preparations that rely solely on S/D treatment for virus inactivation likely should be discontinued. As a consequence of our results, manufacturers of such plasma products were asked to develop and implement virus inactivation steps that are effective against nonenveloped viruses.

Acknowledgments

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References