Different behavior of erythrovirus B19 and torquetenovirus in response to a single step of albumin purification

Alberta Azzi, Fabrizio Maggi, Krystyna Zakrzewska, Maria Carla Menconi, Niccolò Di Pietro, Vittorio Salotti, Claudio Farina, Elisabetta Andreoli, Bruno Fiorentino, Cristina Angelini, Fabiana Corcioli, and Mauro Bendinelli

BACKGROUND: The safety of human serum albumin (HSA) is of special interest with respect to virus transmission because of the wide use of this blood product as a therapeutic agent and also, added to other products, as an excipient or a stabilizer. Conflicting data are reported concerning HSA contamination by small, naked viruses such as the erythrovirus B19 (B19V) and the anellovirus torquetenovirus (TTV). This study has been performed to assess the effect of the HSA purification procedures on the viral contamination.

STUDY DESIGN AND METHODS: Known concentrations of B19V and TTV virus were spiked in raw Fraction V, the starting material from fractionated human plasma for HSA purification, which was subsequently submitted to the depth filtration procedure. After spiking, B19V and TTV genome copies were determined by real-time quantitative polymerase chain reaction assays in the mixture at the end of Fraction V dissolution, to determine the virus concentration achieved, in the HSA solution after the filtration step, in the filtered postwashing fluid, and in the supernatant of resuspended Celite.

RESULTS: B19V was completely adsorbed by the Celite used as a filter aid in the depth filtration process and was thus undetectable in the resulting HSA-containing fraction. In contrast, in 2 out of 3 experiments, TTV was detected in all samples.

CONCLUSION: The different behavior of the two viruses might be a reflection of their different surface charge.

ABBREVIATIONS: B19V = erythrovirus (human parvovirus) B19; TTV = torquetenovirus.
(TTV) are two of such viruses that may be transmitted iatrogenically by blood and blood derivatives. Both are small, naked, single-strand DNA virus. Apart from these shared properties, B19V and TTV are quite different structurally, biologically, and epidemiologically. B19V DNA is linear, whereas TTV DNA is circular. B19V prevalence in blood donations is estimated to range between 1 in 1000 and 1 in 50,000. Thus, the risk of B19 transmission by blood products is dependent on the presence of high virus titers in the infected donors and on the pooling of large number of donations.

In contrast, TTV viremia is widespread among the general population worldwide and its levels vary extensively, ranging between 10^3 and more than 10^8 genome copies per mL in chronically infected individuals. The spectrum of B19V manifestations is wide, ranging from asymptomatic infection to severe diseases and persistent infection, whereas to date there are no clinical manifestations that have been unequivocally linked to TTV.

The safety of human serum albumin (HSA) is of special interest with respect to virus transmission because of the wide use of this blood product as a therapeutic agent in itself and as an excipient or a stabilizer in formulations of a variety of drugs. Several studies have reported the detection of B19V in HSA and other plasma derivatives by polymerase chain reaction (PCR), while others have failed to detect traces of the virus in HSA. In previous studies, we detected TTV but not B19V DNA in some lots of HSA and first-generation recombinant Factor (F)VIII concentrates stabilized with HSA. In contrast, Kreil and colleagues detected no TTV sequences in either HSA or first-generation recombinant FVIII concentrates. Now HSA has been removed from the final formulation of all recombinant FVIII and FIX concentrates. The absence of TTV contamination in HSA was reported also by Pisani and coworkers. These contradictory findings may reflect disparities in the sensitivity of the detection methods used as well as other differences in the study protocols.

More recently, we assessed the effect of HSA purification procedures on B19V and TTV contamination. To this purpose, the entire production method of HSA purification from plasma fractionation was scaled down to give a protein-partitioning profile comparable to that obtained at manufacturing scale. We evaluated the viral removal in each steps of the purification process of HSA and here we particularly focus our attention on the step of depth filtration of dissolved raw Fraction V, the starting material from fractionated human plasma for the purification of albumin. This step, not evaluated in previous studies, has demonstrated an unexpected role in viral removal. Known concentrations of B19V and of TTV were spiked in raw Fraction V, which was subsequently submitted to the deep filtration procedure.

### MATERIALS AND METHODS

#### Depth filtration of solubilized raw Fraction V

Raw Fraction V from fractionated human plasma was added to cold distilled water. The mixture was subjected to mechanical stirring, sustained enough to obtain a complete homogenization, but not too much to avoid foam formation. The temperature was then set at −3 ± 1°C, and the mixture was maintained in these conditions for about 3 days. Celite was added and mixed to the solubilized Fraction V at −3 ± 1°C as filter aid.

A filter (PLAQ, CUNO Inc., Meriden, CT) was washed with EtOH 10 percent, and the suspension obtained was forced to filter through the washed CUNO PLAQ at a pressure of 0.2 to 0.5 bar and at −3 ± 1°C. The filtered product was collected as albumin solution for further analysis. The filter was postwashed with EtOH 10 percent at the same pressure and temperature, and the filtered postwashing solution was collected separately for further analysis (in the production process the albumin solution and the filtered postwashing solution are pooled). The Celite, which is usually a production reject, was removed from the filtering surface and resuspended in phosphate-buffered saline buffer, pH 7.2, stirred overnight. The suspension was then centrifuged, and the supernatant was recovered for further analysis. Three experiments were performed on different days, with three samples from the same batch of raw Fraction V.

#### Virus spiking

Human serum samples containing known numbers of viral genomes and kept in aliquots at −80°C were used as source of B19V or TTV because both viruses fail to grow efficiently in tissue culture. For B19V, serum sample S22, which contained 1 × 10^12 genome copies per mL and no detectable anti-B19V, was used for all the spiking experiments. For TTV, the viremic serum samples were from three healthy individuals and contained 1.4 × 10^6, 1.2 × 10^6, and 4.6 × 10^6 viral genomes per mL, respectively.

These serum samples were not heat-treated to inactivate complements. The genome copy number per mL did not change significantly after DNase I treatment of the four serum samples, suggesting the prevalent presence of complete capsids, protecting the viral DNA, in B19V-positive serum as well as in the three TTV-positive sera. All serum samples were free of hepatitis B and C viruses and human immunodeficiency virus, as determined by specific serologic and molecular assays.

Spiking was carried out during raw Fraction V dissolution, at approximately 1.5 hours from the beginning of the process. Two-milliliter samples of the following materials were collected: Fraction V before spiking, to assess B19V and TTV status of the starting material; Fraction V after spiking, at the end of dissolution, to determine the
virus concentration achieved; postfiltration albumin solution; postfiltration postwashing solution; and supernatant of the resuspended Celite.

**B19V detection and quantification**

DNA was extracted by a DNA-RNA extraction kit (DNA-RNA EXTRA kit, GeneDia, Napoli, Italy) following the manufacturer’s instructions. All the samples were screened for B19V DNA by a one-step PCR procedure that targets a sequence within the genome region coding for the nonstructural protein NS1. The primer pair P1 (5′-ATACACTGTGTGTATGGGCCG-3′, nucleotides 1399-1422) and P6 (5′-CCATTGTGGTTTATAACCACAGG-3′, nucleotides 1682-1704) was used in both the qualitative and quantitative PCR assays. The single-step PCR protocol of two amplification cycles of 1 minute at 95°C, 1 minute at 56°C, and 1 minute at 72°C followed by 40 cycles, each of 30 seconds at 94°C, 30 seconds at 56°C, 30 seconds at 72°C, and a final extension of 10 minutes at 72°C. The B19V DNA–positive samples were then submitted to a quantitative real-time PCR (Real Quant B19 kit, GeneDia), which used SYBR Green as intercalant. Ten microliters of extracted DNA was used in a final reaction volume of 50 µL, following the manufacturer’s instructions. The PCR mixture consisted of 15 µL of RealT-Buffer, 1 µL of each primer P1 and P6 (25 µmol/L), 6 µL of 25 mmol/L MgCl2, 0.5 µL of Taq Gold (5 U/µL; Roche, Indianapolis, IN), and 1 µL of UDG heat label (1 U/µL); water was added to obtain a 40-µL volume. After initial incubation at 20°C for 10 minutes and at 94°C for 6 minutes to activate the Taq polymerase, the thermal conditions followed 2 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, and 40 cycles of 95°C for 1 minute, 55°C for 30 seconds, and 72°C for 30 seconds. Melting curve analysis was performed to assess the specificity of the PCR products (Tm = 81°C) Serial dilutions of the PCR product were examined in triplicate, and at least two independent DNA extractions for each sample were examined.

**RESULTS**

The starting fraction V was found to be free of B19V and TTV, confirming previous findings by us (data not shown) that the two viruses are removed by the fractionation process, particularly during separation of the Supernatant IV, the step that immediately precedes Fraction V production. Nevertheless, considering the possibility that residual amount of either B19V or TTV may still be present in Fraction V, as a consequence of a particularly high viral load in the starting material, we have evaluated also the role of depth filtration of solubilized Fraction V on viral removal.

Spiking was successful for both viruses: in different experiments, contaminating viral genome copies varied between 2.3 × 10^6 and 1.3 × 10^7 for B19V and between 3.0 × 10^5 and 1.7 × 10^6 for TTV. As shown in Tables 1 and 2, measurement of viral contamination in the postspiking treatment was achieved using a real-time PCR assay targeted to a highly conserved segment of the untranslated region of the viral genome, which has the potential to detect with similar efficiencies all of the TTV isolates hitherto recognized. The procedures used for viral genome counting and evaluation of intra- and interassay accuracy and reproducibility have been previously described. The lower limit of sensitivity was 1.0 × 10^3 TTV DNA copies per mL of human serum. All samples were assayed simultaneously in triplicate, and at least two independent DNA extractions for each sample were examined.

**TABLE 1. Summary of B19 and TTV DNA detection in the different steps of depth filtration process during HSA purification**

<table>
<thead>
<tr>
<th>Sample</th>
<th>B19 virus</th>
<th>TTV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction V postspiking</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Albumin solution</td>
<td>0/3</td>
<td>2/3</td>
</tr>
<tr>
<td>Postwashing solution</td>
<td>0/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Celite resuspension</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

**TABLE 2. Mean percent of initial viral load found at different steps of depth filtration process during HSA purification**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percent of initial viral load*</th>
</tr>
</thead>
<tbody>
<tr>
<td>B19 virus</td>
<td>67 ± 9</td>
</tr>
<tr>
<td>TTV</td>
<td>18 ± 9</td>
</tr>
<tr>
<td>Albumin solution</td>
<td>0</td>
</tr>
<tr>
<td>Postwashing solution</td>
<td>0</td>
</tr>
<tr>
<td>Celite resuspension</td>
<td>100</td>
</tr>
</tbody>
</table>

* Mean of three experiments for B19 virus and two experiments for TTV.

**TTV detection and quantification**

DNA was extracted from 200 µL of sample with a DNA extraction kit (QIAamp DNA blood mini kit, Qiagen, Chateloth, CA). TTV DNA detection and quantification was carried out with a TaqMan real-time PCR assay targeted to a highly conserved segment of the untranslated region of the viral genome, which has the potential to detect with similar efficiencies all of the TTV isolates hitherto recognized. The procedures used for viral genome counting and evaluation of intra- and interassay accuracy and reproducibility have been previously described. The lower limit of sensitivity was 1.0 × 10^3 TTV DNA copies per mL of human serum. All samples were assayed simultaneously in triplicate, and at least two independent DNA extractions for each sample were examined.
samples revealed an interesting divergence between B19V and TTV. B19V was constantly undetectable in the albumin solution, as well as in the filtered postwashing solution. Furthermore, all the B19V added was recovered in the fluid used to wash Celite after its use for filtration. In contrast, in two of three experiments, TTV DNA was present in all the postfiltration samples (Table 1) and most of this virus (mean, 67% of the total virus added) was in the albumin solution (Table 2). In the third experiment, the albumin solution yielded no detectable TTV, albeit the virus was detected in all other samplings.

Further experiments were then performed to ascertain whether the different behavior of B19V and TTV could be due to a different ability to interact with Celite. Because the S22 serum sample used for the B19V spiking was free of anti-B19 antibody, whereas the serum sample used for TTV spiking contained anti-TTV activity as determined by immunoprecipitation (data not shown), we considered that the presence of virus-antibody complexes might reduce the ability of Celite to adsorb the corresponding virus. To verify this hypothesis, the S22 serum sample was mixed with a B19 DNA–negative and anti-B19 IgG highly positive serum sample for 2 hours at room temperature and then the mixture was used for B19V spiking. Dissolved Fraction V spiked with both B19V and TTV in immunocomplexed form was mixed and kept in contact with Celite. As shown in Table 3, examination of Celite samples and Celite supernatants taken at different time points showed that also under these conditions Celite was capable of effectively adsorbing B19V but not TTV, thus excluding that the different behavior of B19V and TTV was attributable to the presence of immunocomplexed virus in the later.

Recently Boschetti and colleagues showed that B19V is inactivated by incubation at pH 4 for 2 hours. It was thus conceivable that the nearly 3 days of maintenance at pH 4.8 needed for Fraction V dissolution influenced the integrity of B19V virions more severely than that of TTV virions, thus differently affecting the recovery of the two viral genomes. To test this possibility, the Celite resuspensions B19 DNA–positive samples and the supernatants TTV DNA–positive samples were treated with a high concentration of DNase I (200 units/mL; Roche) for 2 hours and then examined for viral DNA content. This treatment did not significantly affect the amount of B19 DNA detectable in the Celite suspension and of TTV DNA in the supernatant. As controls, B19 DNA and TTV DNA extracted from B19V- and TTV-positive serum samples, respectively, added to the same materials were completely digested by the DNase treatment.

**DISCUSSION**

In these experiments we investigated whether B19V and TTV are removed differently from spiked Fraction V by the process of Celite filtration. The study was prompted by conflicting reports regarding the presence of B19V and TTV in commercial HSA.

The numbers of B19V genomes per mL of solubilized Fraction V obtained by spiking were compatible with the ones that one can expect in plasma pools containing a donation with high B19V titer (Gallinella et al. and our own data). It is, however, important to emphasize that it is highly unlikely that a similar B19V load is present in Fraction V, owing to the fact that standard plasma fractionation procedures leading to this product greatly, if not completely, remove this virus. Nevertheless, the present results demonstrate that B19V is completely adsorbed to the Celite used for the depth filtration step for HSA purification, being undetectable also in the postwashing solution. Thus, any residual B19V that might remain in Fraction V would be effectively removed from the fraction containing this protein. In contrast, removal of TTV by Celite filtration was much less efficient. Indeed, TTV was not significantly absorbed by Celite, regardless of the duration of contact.

Experiments showed that the observed different behavior of the two viruses was not attributable to the circumstance that the TTV-positive serum samples used for spiking contained immunocomplexed virions, whereas the serum sample used for B19V spiking did not, nor to a differential effect of the relatively low pH used for dissolving Fraction V on the accessibility of viral DNA to digestion by nuclease. It seems therefore likely that the different behavior is at least in part due to differences in the surface charge of the two viruses; in fact, the calculated isoelectric point of the B19V capsid proteins (both VP1 or VP2) varies between 6.17 and 6.40 whereas that of the TTV capsid protein (ORF I) varies between 10.52 and 10.87 depending on viral genotype (Takahashi et al. and our own data). This explanation is in line with additional findings of ours pointing out that, during the complete
fractionation process, when Celite filtration is preceded by the change of some variables such as EtOH concentration, ionic strength, or pH, the two viruses exhibit the same behavior, sorting out in the same way in the Celite or in the product of filtration (data not shown). We could argue from this that parameter variations modify the capsid proteins in such a way that the two viruses behave similarly. When the filtration is only preceded by dissolution of the starting Fraction V, as is the case in the present study, viral behavior is determined by the native properties of the capsid proteins.

In conclusion, these results confirm that it is highly unlikely to find B19V in HSA prepared by the procedure we have used because the single step of Celite filtration is sufficient to bring this virus under detection levels. Reports by others that B19V can be detected in certain HSA batches may be due to the use of different overall procedures by the manufacturers. In any case, a small residual amount of B19 virus should be rapidly inactivated during pasteurization of HSA, as recently reported. Celite filtration is instead insufficient to clear TTV from HSA, albeit this goal could be probably achievable with the implementation of additional purification steps. Altogether these results underline that a similar procedure may remove certain viruses but may be ineffective against others, even when the viruses in question have essentially similar structural features. In accordance with standard process validation guidelines, these observations support the importance of monitoring the viral removing ability of each individual step that is or may be employed in the purification of plasma derivatives by use of a wide spectrum of potential viral contaminations, including emerging and reemerging viruses and, above all, prions.

REFERENCES
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