CONCISE COMMUNICATION

Evidence of Porcine Endogenous Retroviruses in Porcine Factor VIII and Evaluation of Transmission to Recipients with Hemophilia

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Since 1984, unheated porcine clotting factor VIII (Hyate:C) has been used to treat severe bleeding episodes in persons with hemophilia who have antibodies to human clotting factor. We document the presence of porcine endogenous retrovirus (PERV) in plasma samples of pigs and in clinical lots of Hyate:C. Both gag and pol PERV RNA sequences were detected by reverse-transcriptase (RT) polymerase chain reaction in 13 of 13 lots of Hyate:C tested. Among 10 of these lots, RT activity also was detected, which confirms the presence of retroviral particles. To assess the transmission of PERV to Hyate:C recipients, we tested serum specimens from 88 recipients of Hyate:C and 23 noninfused control subjects for anti-PERV antibodies by using a Western blot assay. None of the samples was positive. Our data document that PERV particles are a common contaminant of Hyate:C products and suggest that the risk of PERV transmission from these percutaneous exposures is very low.

The reliance of persons with hemophilia on parenterally administered clotting factors has resulted in exposure to infectious agents that can be present in blood-derived products. The transmission of human immunodeficiency virus type 1 (HIV-1) to approximately half the hemophilia population before the availability of a screening assay illustrates the tragic consequences of contaminated blood products on this population [1]. A number of measures have been implemented in recent years, to ensure the continued safety of plasma derivatives. These measures include screening of blood donors, incorporation of virus inactivation or removal into the manufacturing process, and development of recombinant factor concentrates [2].

Approximately 8%–10% of patients treated with human factor VIII develop persistent inhibitors (antibodies) that render these concentrates ineffective for controlling bleeding episodes [3]. Porcine factor VIII concentrate (Hyate:C), which is purified from pooled plasma of farm-raised pigs, lacks antibody-specific epitopes and has been used successfully to treat patients with inhibitors [3, 4]. For many patients, Hyate:C may represent a life-saving alternative in many cases [3].

The use of Hyate:C has the potential to expose human recipients to pig-derived infectious agents. Current manufacturing practices include rigorous screening of source animals, plasma, and final products to reduce or eliminate contamination with known infectious agents [5]. Screening of source animals, however, cannot eliminate one group of viruses, the porcine endogenous retroviruses (PERVs), because they are expressed from DNA elements present in the genomes of all pigs [6–8]. Infectious PERV is released in vitro from a variety of porcine cells, including peripheral blood mononuclear cells (PBMC) and certain kidney cell lines [6, 8].

Concerns regarding transmission of PERV to humans have been widely discussed in exposures related to the use of pig tissues as xenografts in humans [9]. These concerns were heightened when PERVs derived from primary porcine PBMC and other pig cell cultures were shown to be infectious for some human cells [6–8]. Although PERV appears to be nonpathogenic in pigs, its potential to infect humans and to cause disease remains unknown. PERV has 3 envelope classes (PERV-A, -B, and -C) that have been shown to have distinct receptor specificity and in vitro tropisms [7, 9]. Both PERV-A and PERV-B have been shown to infect human cells [7, 9].

PERV is not latent in vivo, and evidence of both PERV RNA and reverse-transcriptase (RT) activity has been detected in pig serum samples, which suggests productive release of PERV particles into the circulation [10]. The detection of PERV in pig serum samples raises the possibility of finding it in products derived from porcine plasma. Screening such products for PERV is not addressed in current manufacturing guidelines, because these viruses were not known to be present in pig plasma. In this study, we report evidence of PERV in Hyate:C.
and investigate transmission of PERV to recipients of this product.

Materials and Methods

**RT–polymerase chain reaction (PCR) testing for PERV RNA in pig serum/plasma samples and Hyate:C.** Eight plasma and 12 serum samples from pigs were obtained from White Landrace x Duroc x Hampshire. Thirteen lots of previously released Hyate:C sera were reconstituted according to package inserts and were stored at −20°C before testing. RT-PCR for PERV RNA was done, as described elsewhere [10, 11]. RNA extracts were digested with DNaSe-I (Boehringer-Mannheim), followed by the inactivation of the DNaSe by boiling for 5 min [10, 11]. RNA extracts corresponding to 50 μL of Hyate:C or 50 μL of pig plasma and serum samples were tested for PERV gag and pol RNA sequences, as described elsewhere [10, 11]. PCR assays that omitted RT were included for each sample, to confirm that a positive result was due to the presence of PERV RNA and was not the result of contamination with residual PERV DNA. Detection of PCR products was done by Southern blot hybridization to 32P-labeled internal oligoprobes [10, 11]. Positive controls included PERV RNA extracts from tissue culture supernatants from the PK15 or Shimozuma-1 cell lines.

**Screening for RT activity in pig serum and plasma samples and in Hyate:C products.** RT activity in serum and plasma samples was detected by using the PCR-based Amp-RT assay in duplicate on ultracentrifuged pellets that are equivalent to 10 μL of serum and/or plasma, followed by Southern blot hybridization, as described elsewhere [10]. Positive controls were tissue culture supernatants containing PERV and HIV-1, and negative controls included water and human serum samples that are antibody negative for all known human retroviruses. Ten microliters of Hyate:C were screened initially for RT activity by Amp-RT. Hyate:C samples that had negative Amp-RT results were tested further, to exclude the possibility of assay inhibition. Screening for assay inhibition in Hyate:C products was done by Amp-RT testing of products spiked with either PERV or HIV-1. Spiked samples that showed evidence of Amp-RT inhibition then were analyzed by several protocols, including direct testing of various dilutions of Hyate:C (from 1:2 to 1:100) and by testing of various dilutions of ultracentrifuged Hyate:C pellets.

**Clinical specimens.** A total of 111 serum samples from persons with hemophilia were screened for PERV antibodies. These samples included 88 from Hyate:C recipients and 23 from nonrecipients. All 111 patients were identified from among 134 federally funded hemophilia treatment centers (HTCs) in the United States and 4 HTCs in Canada, as described elsewhere [5]. The Hyate:C recipients included persons who had been infused with this product from 1 January 1992 through 15 October 1996. The number of different lots received per person was 1–6 lots, with an average of 2 lots.

**PERV antibody testing.** Testing for antibodies to PERV was done in a blinded fashion regarding Hyate:C exposure status and by using a previously developed Western blot assay [10, 12]. This assay uses whole-cell lysates derived from PERV-infected human embryonic kidney 293 cells (kindly provided by Robin A. Weiss, University College London, London, United Kingdom) as a source of PERV antigen. Serum samples were tested at 1:50 dilution. Antibody reactivity to p30 gag PERV protein was visualized by chemiluminescence, using ECL Western blotting reagents (Amersham), as described elsewhere [12].

**PERV envelope subtype analysis.** To type PERV envelope sequences, RNA from Hyate:C was analyzed by using PCR amplification and sequence analysis. PERV-A and PERV-C subtypes were amplified generically by RT-PCR amplification with PENVF1 (forward) 5'-TCTTTAGGAACCTGGTGCCCTGA'3' and PENVR1 (reverse) 5'-TCTCAGTGAACATTTTTAGTAATCTTAG-G(A/G)GTCT-3'. Negative controls (N/N) were used to accommodate nucleotide variability. PCR products were cloned into the pcR 2.1 vector (Invitrogen), and multiple clones were sequenced and were subtyped on the basis of the homology to prototype PERV-A (EMBL Y12238), PERV-B (Y12239), and PERV-C (AF038600 and AF038599) env sequences. Screening for PERV-B was made with PERV-B-specific primers PL 172 and 173, as described elsewhere [7].

**Results**

**Screening for PERV RNA in pig serum and plasma samples and Hyate:C products.** Of the 20 pig serum and plasma samples examined for the presence of PERV gag RNA by RT-PCR, 17 (85%) were positive, including all 8 plasma samples and 9 of the 12 serum samples (figure 1A). The results for the 12 serum samples have been reported elsewhere by us [10]. Contamination with residual PERV genomic DNA was excluded, because control RT-PCR assays done without RT were all negative (figure 1A). These results confirm the release of PERV into the plasma or serum samples of the majority of pigs. All 13 Hyate:C lots were positive for both PERV gag and pol RNA sequences by RT-PCR (figure 1B). In all amplifications, the PCR products could be easily visualized with ultraviolet light in ethidium bromide-stained agarose gels before Southern blot transfer and hybridization, which suggests that viral RNA is present at relatively high levels. Contamination with PERV genomic DNA was also excluded in all Hyate:C because of the negative results found with the control reactions (figure 1B).

**Screening for RT activity in pig serum and plasma samples and Hyate:C products.** To confirm the presence of retroviral particles in either pig serum/plasma samples or Hyate:C products, we tested them all for RT activity, which is associated with retroviral particles. Of the 20 serum and plasma samples, 16 (80%) serum and plasma samples were Amp-RT positive. All 16 serum and plasma samples were determined to be PERV RNA positive by RT-PCR (figure 1C). Of the 13 lots of Hyate:C, 10 had detectable RT. Some Hyate:C lots had false-negative results in the initial screening because of variable levels of assay inhibition; however, detectable Amp-RT activity was seen by additional testing of pellets or of diluted products. Figure 1C shows representative data from duplicate test results.

**PERV antibody testing.** All 111 samples, including the 88 Hyate:C recipients and the 23 nonrecipients, were neg-
Figure 1. Screening of pig plasma and serum samples and porcine factor VIII concentrates (Hyate:C) for porcine endogenous retrovirus (PERV) RNA and reverse-transcriptase (RT) activity. A, RT–polymerase chain reaction (PCR) signals of PERV gag RNA of pig plasma and serum samples. B, PERV gag RNA signals from Hyate:C lots. C, Duplicate test results for RT activity in pig plasma and serum samples and Hyate:C. HIV, human immunodeficiency virus; NHS, normal human serum; –RT, signals from control RT-PCR reactions done without reverse transcription; +RT, signals from test sample reactions with reverse transcription.

Discussion

This study demonstrates that porcine factor VIII concentrates contain both PERV RNA and RT activity. The finding that all lots were determined to be positive for PERV RNA indicates that PERV is a common contaminant of Hyate:C products. The ubiquitous presence of PERV in Hyate:C is not surprising and can be explained by our findings of release of PERV into the plasma samples of all pigs tested. The presence of PERV in pig plasma samples is consistent with both the
endogenous presence of PERV DNA in all porcine cells and the evidence of PERV expression seen in various pig tissues [6–8]. Because porcine factor VIII is derived from pooled plasma, it is, therefore, very unlikely that any batch of porcine factor VIII concentrate would be free of PERV.

Because the usual dose of Hyate:C for patients with hemophilia involves multiple vials and because many patients receive material from many lots, it is possible that exposure to PERV in recipients was universal [5]. Despite these parenteral exposures, we found no serological evidence of PERV infection in all 88 Hyate:C recipients tested. The negative antibody results are significant, because they reflect absence of antigenic exposure due to virus replication that occurs in different cellular and tissue compartments. These findings suggest that patients receiving Hyate:C are at a very low risk of infection with PERV.

About 13% of the Hyate:C recipients in this study were infected with HIV-1, primarily by previous exposures to unheated HIV–1–contaminated human factor VIII. Infection with HIV-1 occurred in the hemophilia population, despite both the generally low prevalence of HIV-1 RNA in unheated human factor VIII batches and the inability to isolate HIV-1 from these products [13, 14]. Therefore, the lack of transmission of PERV to Hyate:C recipients, despite the apparent universal exposure, may reflect a lower transmissibility of this virus, compared with that of HIV-1. Host factors may also play a role. For example, PERV is susceptible to complement-mediated lysis from human serum [6]. This observation is most probably due to complement activation by naturally occurring antibodies to the α-1,3 galactose residues present on pig cell–derived PERV envelopes. Therefore, cell-free PERV present in Hyate:C preparations may be rapidly removed by this mechanism after parenteral injection of Hyate:C. Rapid clearance of PERV would probably explain the absence of a specific anti-PERV antibody response in these patients, despite exposure to PERV particles.

Although no virucidal treatment is incorporated in current Hyate:C production, existing steps for physical purification and concentration, including filtration, polyethylene glycol precipitation, and lyophilization, may all reduce the infectious titer of PERV to a level that may be insufficient to produce infection in Hyate:C recipients. In a study of human factor VIII spiked with a murine retrovirus, lyophilization has been demonstrated to reduce infectious titers by 100-fold [15]. The observed predominance of PERV-C in the Hyate:C lots analyzed also could reduce transmissibility to humans, because, unlike PERV-A and PERV-B, these viruses are not known to infect human cells [7, 8]. The predominance of PERV-C also suggests that culture systems using cells that are permissive to PERV-C infection will be required to investigate the presence of infectious PERV in Hyate:C.

Our findings suggest that PERV inevitably contaminates porcine factor VIII products that are currently prepared without specific PERV removal or inactivation steps; however, the absence of PERV transmission in all 88 Hyate:C recipients is reassuring and supports the continued use of this life-saving blood product. The addition of virucidal steps, such as heating, solvent detergent treatment, or low pH to the manufacturing process, currently under evaluation by the maker of this product, could reduce further the very low potential risk for PERV transmission.

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References