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No Evidence of Xenotropic Murine Leukemia Virus-Related Virus Transmission by Blood Transfusion from Infected Rhesus Macaques

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The discovery of xenotropic murine leukemia virus-related virus (XMRV) in human tissue samples has been shown to be due to virus contamination with a recombinant murine retrovirus. However, due to the unknown pathogenicity of this novel retrovirus and its broad host range, including human cell lines, it is important to understand the modes of virus transmission and develop mitigation and management strategies to reduce the risk of human exposure and infection. XMRV transmission was evaluated by whole-blood transfusion in rhesus macaques. Monkeys were infected with XMRV to serve as donor monkeys for blood transfers at weeks 1, 2, and 3 into naïve animals. The donor and recipient monkeys were evaluated for XMRV infection by nested PCR assays with nucleotide sequence confirmation, Western blot assays for development of virus-specific antibodies, and coculture of monkey peripheral blood mononuclear cells (PBMCs) with a sensitive target cell line for virus isolation. XMRV infection was demonstrated in the virus-injected donor monkeys, but there was no evidence of virus transmission by whole-blood transfusion to naïve monkeys based upon PCR analysis of PBMCs using XMRV-specific *gag* and *env* primers, Western blot analysis of monkey plasma up to 31 to 32 weeks after transfusion, and coculture studies using monkey PBMCs from various times after transfusion. The study demonstrates the lack of XMRV transmission by whole-blood transfusion during the acute phase of infection. Furthermore, analysis of PBMC viral DNA showed extensive APOBEC-mediated G-to-A hypermutation in a donor animal at week 9, corroborating previous results using macaques and supporting the possible restriction of XMRV replication in humans by a similar mechanism.

The initial discovery of xenotropic murine leukemia virus-related virus (XMRV) in human prostate cancer tissue (1), and later in some peripheral blood mononuclear cell (PBMC) samples from patients with myalgic encephalomyelitis/chronic fatigue syndrome (2), raised public health concerns related to the discovery of a novel human retrovirus and potential virus transmission due to exposure to mice (3) and infected blood donors and blood-derived products (4–11). These emerging concerns led to intensive discussions and investigations of XMRV, including molecular and biological characterization of the virus and the development of assays, standards, and nonhuman primate (NHP) models for further studies of human infection and disease association. The results of several studies evaluating XMRV infection in humans indicated that the results of the original reports were due to sample and/or laboratory contaminations (12–19). Furthermore, XMRV was found at high titers in the 22Rv1 human prostate cancer cell line (20) and was recently shown to have most likely originated from recombination between two different endogenous murine retrovirus sequences during derivation of the 22Rv1 human prostate cancer cell line by serial passage of a human prostate tumor xenograft in nude mice (21, 22). These findings have led to the general scientific consensus that XMRV is not a human retrovirus but a novel recombinant murine retrovirus with some unique biological properties (23). XMRV has a broad host range and can infect a variety of human cell lines *in vitro* (24–27) and nonhuman primate cells and tissues *in vivo* (5, 28). Studies of XMRV injection in rhesus macaques and pigtailed macaques along with a study of wild-derived *Mus pahari* mice (29) indicated that XMRV infection shows a transient acute phase of infection, during which time the virus was detected in peripheral blood cells. After this phase, however, the virus could not be detected in the blood but persisted at low levels in various host tissues. Additionally, a low level of vertical transmission was shown in the mouse study (30).

An important aspect of biological products is to demonstrate the absence of unintended viruses and to determine the risk of human infection and virus transmission in case of inadvertent exposure and infection. Due to the undefined pathogenic potential of XMRV, the unexpected discovery of the virus or its sequences in some cell lines used broadly in research, and broad contamination of laboratory reagents with murine leukemia virus (MLV)-related sequences, it is prudent to evaluate the presence of XMRV in biological materials used for manufacturing of products for human use. XMRV has been investigated and was shown to be absent in live-virus vaccines (31), and we previously developed sensitive PCR assays and demonstrated the absence of XMRV-specific sequences in a variety of cell lines, including some related to vaccine cell substrates (32). In this study, we have used the rhesus macaque model to evaluate the modes of XMRV transmission by investigating virus infection and replication after direct virus injection or blood transfusion from infected monkeys.

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MATERIALS AND METHODS

XMRV stock. XMRV stock was prepared by transfection of LNCaP cells (human prostate carcinoma cell line; ATCC CRL-1740, clone FGC) with VP62/pcDNA3.1 (contributed by R. H. Silverman and B. Dong and obtained from the NIAID AIDS Repository), using Lipofectamine 2000 (catalogue no. 11668-019; Invitrogen, Carlsbad, CA). Briefly, 500 μl RPMI 1640 medium (catalogue no. 112-024-101; Quality Biologicals) containing 5 μg DNA was combined with 500 μl RPMI 1640 medium (containing 20 μl Lipofectamine 2000 and incubated for 15 min at room temperature before being added to LNCaP cells [400,000 cells, which were planted 24 h prior to transfection in a 25-cm^2 flask]). After 16 h of incubation at 37°C, RPMI 1640 medium was changed to RPMI 1640 complete medium (supplemented with 10% fetal bovine serum [FBS], heat inactivated at 56°C for 30 min [catalogue no. SH30071.03; HyClone, Logan, UT]; 2 mM L-glutamine; 1 mM sodium pyruvate; 10 mM HEPES [catalogue no. 15630-080; Invitrogen, Carlsbad, CA]; 250 U of penicillin per ml; and 250 μg of streptomycin per ml). Cells were transferred into a 75-cm^2 flask on day 3 and then into a 150-cm^2 flask on day 19. Cells were passaged at a subcultivation ratio of 1:3 every 4 days upon reaching confluence using 0.05% trypsin–0.53 mM EDTA (catalogue no. 25300-054; Invitrogen). Medium was changed on day 29 and reduced from 40 ml to 25 ml for preparation of the virus stock on day 30 by pooling and filtering the supernatant (0.45-μm filter; Corning) and storing aliquots of the XMRV stock at −80°C. Virus production in the supernatant was determined using the single-tube fluorescent product-enhanced reverse transcriptase (RT) (STF-PERT) assay (33). Cells at day 34 were collected along with uninfected control cells and prepared for transmission electron microscopy (TEM) analysis, as previously described (34). The XMRV stock titer (50% tissue culture infective dose [$TCID_{50}$] endpoint) was determined in LNCaP cells and in Mv1Lu cells (mink lung; ATCC CCL-64) using 10-fold serial dilutions and evaluation of the PERT activity in filtered supernatants collected at day 7. The $TCID_{50}$ was calculated by the Karber method (35). Mv1Lu cells were grown in Eagle's minimum essential medium (modified) with Earl's salt without L-glutamine (catalogue no. 15-010-CV; Mediatech, Manassas, VA) containing 10% FBS, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml with 1× nonessential amino acids (MEM-NEAA, 100×; Quality Biological, Inc., Gaithersburg, MD) and 1 mM sodium pyruvate (designated complete EMEM). Additionally, virus focus-forming units (FFU) per ml were determined by a mink S^+ L^- assay (BioReliance).

Monkey injections and blood transfusions. The source of the animals, retrovirus screening, and maintenance of rhesus macaques (*Macaca mulatta*) at the FDA animal facility (National Institutes of Health, Bethesda, MD) were previously described (36). The animals, designated DBL2, DBNP, DBHH, DBHE, DBLZ, DBCF, and DBHT, were adults at the time of this study and had previously tested negative for type D simian retrovirus (SRV), simian T-cell lymphotropic virus (STLV), and simian immunodeficiency virus (SIV) based upon serology and further tested negative for SRV based upon a PCR assay and virus isolation. All animals were positive for simian foamy virus (SFV) by a virus-specific, nested PCR assay described previously (34), except animals DBL2 and DBHE, which were negative based upon a PCR assay and by a lack of virus isolation using monkey PBMCs in a coculture assay. Prior to the testing and subsequently, the animals were housed in single cages and handled with special precautions to avoid cross-contamination. Rhesus macaque CF86 was added during the course of the study and was SFV positive. All animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* under an approved protocol by the Institute Animal Care and Use Committee (37).

Monkeys were injected with XMRV intravenously (saphenous vein) to serve as donor animals for whole-blood transfusion studies or with medium as a control. For ease of monkey identification in this paper, blood donor animals are indicated with a "(d)" after their designation, blood recipient animals are indicated with an "(r)," and the control animal is indicated with a "(c)." Animals DBL2(d) and DBNP(d) were injected with XMRV stock (1 ml), and animal DBCF(d) was injected with 1 ml of resuspended and pooled virus pellets after ultracentrifugation to remove spent medium (four 1-ml aliquots of the virus stock were ultracentrifuged through 1 ml of 20% sucrose in phosphate-buffered saline [PBS] [pH 7.4] [Quality Biologicals] at 36,000 rpm for 2 h at 4°C using a Beckman TLA45 rotor; each tube was resuspended in 350 μl of complete RPMI medium and pooled). As a control, animal DBHH(c) was injected with complete medium.

Blood (10 ml) was collected in heparin from animals DBL2(d) and from animal DBNP(d) at week 1 after XMRV injection and transfused into animals DBHE(r) and DBLZ(r), respectively. Blood (10 ml) collected in heparin from animal DBCF(d) was transfused at week 2 and week 3 after XMRV injection into animals DBHT(r) and CF86(r), respectively. This is shown schematically in Fig. 2. Blood transfer, collections, and preparation of PBMCs were done as previously described (36).

Blood was collected into EDTA Vacutainer tubes (BD) to prepare PBMCs and plasma, and aliquots were stored in liquid nitrogen and at −80°C, respectively.

Western blot analysis. Cell lysates were prepared from uninfected mink cells and XMRV-infected mink cells as described previously (34). Protein concentration was determined with a protein assay dye (Bio-Rad, Hercules, CA). One hundred twenty micrograms of uninfected or XMRV-infected mink cell lysate was analyzed on a 12% Tris-glycine gel, run for 1.5 h at 125 V (Novex X-cell II system; Novex, San Diego, CA) separately in single-well gels in 1× Tris-glycine running buffer (24.8 mM Tris, 192 mM glycine, 0.1% sodium dodecyl sulfate). Proteins on the gel were transferred onto a nitrocellulose membrane for 1 h at 30 V in a separate apparatus in a solution containing 24.8 mM Tris, 192 mM glycine, and 20% methanol. The membrane was rinsed in transfer buffer and cut into twelve 5-mm strips. The strips were placed protein side up into individual wells of a plastic tray, rinsed at room temperature for 5 min in 5 ml of ultrapure water and PBS without Ca^{2+} and Mg^{2+}, and then blocked overnight on a shaker at 4°C in PBS (pH 7.3)–0.05% Tween (PBST) containing 5% nonfat dried milk (PBST+5%). The strips were incubated at room temperature on a shaker for 4 h. Strips were then incubated with a 1:100 dilution of monkey plasma in PBST+5% for 2 h at room temperature and then overnight at 4°C on a shaker. Strips were brought to room temperature, and unbound antibody was washed three times for 5 min in PBST+5% using approximately 5 ml per strip.

Horseradish peroxidase (HRP)-labeled goat anti-monkey IgG (catalogue no. 074-11-021; Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) was used as a secondary antibody. Strips were incubated in 2 ml each of a 1:40,000 dilution of secondary antibody in PBST+5%, in all cases except for animal DBLZ(r), where a 1:20,000 dilution was used due to low signal, for 2 h on a shaker at room temperature. Strips were then washed four times for 10 min in PBST and once in PBS without Ca^{2+} and Mg^{2+} for 30 min, using approximately 5 ml of solution per strip.

The protein bands were visualized by chemiluminescence with a substrate system (SuperSignal West Pico chemiluminescent substrate; Pierce, Rockford, IL) on a shaker using 2 ml of substrate per filter for 2 min and then blotted with paper (Whatman 3MM; Whatman plc, Maidstone, Kent, England) to remove excess substrate and exposed for various times ranging from 1 s to 6 min using BioMax film (BioMax MR film; Kodak, Rochester, NY).

Infectivity studies. Monkey PBMCs (4×10^6 to 5×10^6 PBMCs) were stimulated with phytohemagglutinin (PHA; catalogue no. HA16; Remel, Inc., Lenexa, KS), as previously described (36, 38), for 72 h in a 25-cm^2 flask prior to coculture with Mv1Lu cells (400,000 cells that were preplanted for 2 h in a 25-cm^2 flask for attachment) in complete EMEM for at least 30 days or until extensive cell lysis occurred due to the cytopathic effect (CPE) of SFV, which was present in some monkeys prior to the study. PBMCs were fed back to the Mv1Lu cells for the initial 3 passages to provide an extended coculture of the PBMCs with the target cells and possibly enhance virus isolation. DNA was prepared from the cells at various times after coculture for PCR analysis. XMRV identity was con-

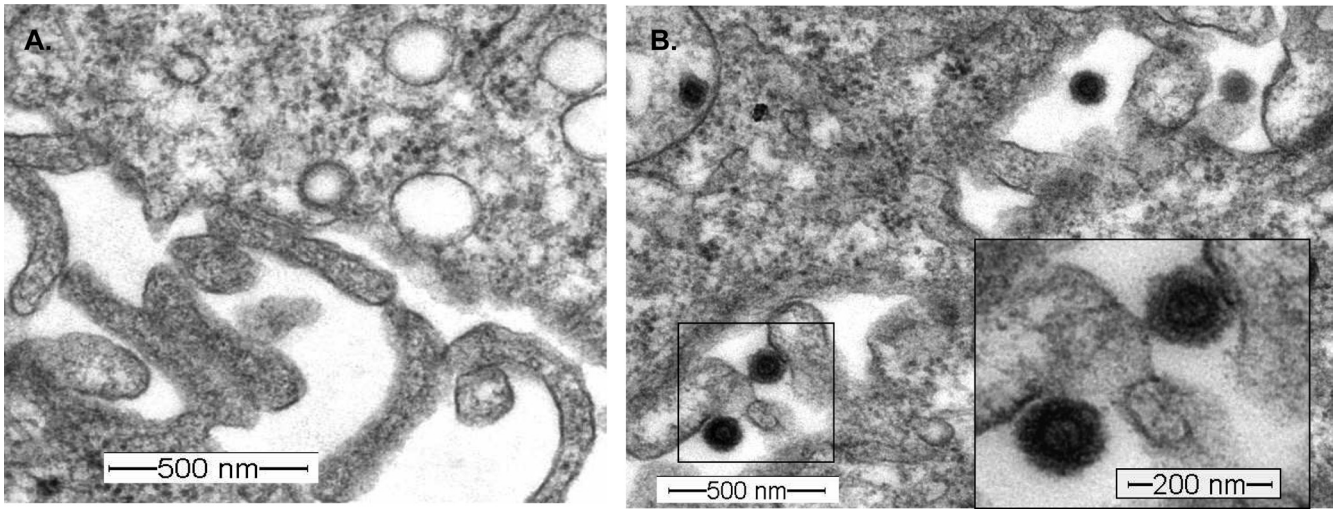

FIG 1 TEM analysis of XMRV-infected cells used for preparation of virus stock. Control LNCaP cells (A) and XMRV-infected LNCaP cells (B) are shown. The boxed area in panel B is enlarged in the inset.

firmed by nucleotide sequence analysis of PCR-amplified fragments. Cell pellets were also prepared for TEM analysis.

Sensitivity of XMRV detection in Mv1Lu cells was determined using 10-fold serial dilutions of the virus stock. Infections were set up by overnight incubation in complete medium containing 4 μg/ml Polybrene (catalogue number TR-1003-G; Millipore, Billerica, MA), after which cells were propagated in complete medium. Cell pellets (2×10^6 cells) were collected at each passage, and DNA was prepared according to the manufacturer's protocol, except that a 10-min incubation at 95°C was added following the proteinase K incubation step (QiaAmp DNA blood minikit; Qiagen, Valencia, CA). Virus replication was evaluated by PCR analysis of 0.67 μg DNA, calculated based upon 6.6 pg DNA per cell, using XMRV *gag* and *env* outer and inner primer pairs. The results were obtained by electrophoresis on a 1.4% agarose gel with UV visualization of ethidium bromide-stained DNA.

PCR assays. Total DNA was prepared directly from whole blood using the QiaAmp DNA blood minikit (Qiagen) as described above or from PBMCs as previously described (36) for PCR analysis. XMRV PCR primers and assays conditions for amplification of *gag* and *env* sequences using outer and inner primers were previously described, with an annealing temperature of 63°C used for the outer primers (32). Detection was based upon UV visualization of amplified fragments in an ethidium bromide-stained gel. The sensitivity of the XMRV PCR assays was determined to be <10 copies in approximately 1.8×10^5 cells, equivalent of human DNA. SFV long terminal repeat (LTR) outer primers were used as previously described (36). Human β-actin primers were used to amplify an 838-bp fragment as a control for the presence of DNA in the sample (Clontech, Mountain View, CA). The forward primer sequence was 5′-ATCTGGCA CCACACCTTCTACAATGAGCTGCG-3′, and the reverse primer sequence was 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′. Thermal cycling conditions were 95°C for 3 min followed by 35 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min. Fragment sizes were determined using a 100-bp ladder (New England BioLabs, Ipswich, MA). PCR without DNA was used as a negative control.

DNA cloning and sequencing. For direct sequencing of DNA, PCR-amplified fragments were treated with ExoSAP-IT (Affymetrix, Santa Clara, CA), and nucleotide sequences were determined with PCR primers by using an Applied Biosystems 3130xl Genetic Analyzer according to the manufacturer's standard protocol (Applied Biosystems, Foster City, CA). Sequence alignment was done using Vector NTI software (Invitrogen, Carlsbad, CA). For single-copy sequencing, PCR-amplified DNA fragments were gel purified using the Zymoclean gel DNA recovery kit (Zymo Research Corporation, Irvine, CA) and cloned by ligation into the pGEM-T Easy vector (Promega Corporation, Madison, WI), according to manufacturer's instructions. Ten colonies were selected from each transformation for DNA preparation using the QIAprep Spin Miniprep kit (Qiagen), and nucleotide sequences were obtained with vector-specific SP6 and T7 primers using the ABI 3130xl Genetic Analyzer. Sequence alignment was done using Vector NTI with XMRV VP62, the inoculating virus, as a reference sequence (GenBank accession no. EF185282).

RESULTS

XMRV infection in rhesus macaques. A well-characterized XMRV stock was prepared by transfection of LNCaP cells with cloned VP62/pcDNA3.1 DNA. XMRV production in the cells was visualized by TEM (Fig. 1), the amount of virus in the stock was quantified for the total number of particles by using an STF-PERT assay, and infectious particles were determined as $TCID_{50}$ in LNCaP cells and Mv1Lu cells. The total number of RT-containing particles determined by the STF-PERT assay was 5.5×10^6 particles per ml; the infectious particles were determined by virus titration using the STF-PERT assay for readout ($10^{5.5}$ $TCID_{50}$ per ml in Mv1Lu cells and $10^{4.5}$ $TCID_{50}$ per ml in LNCaP cells) or focus formation in S^+ L^- mink cells (1.04×10^4 FFU per ml). These results indicated that the ratio of noninfectious to infectious particles was about 10:1 or 100:1, which is the expected range for gammaretroviruses and therefore suitable for use in further studies. Additionally, Mv1Lu cells were found to be 10-fold more sensitive for XMRV replication than LNCaP cells and were a better target cell line for virus detection and isolation.

Two studies were done to evaluate infection of rhesus macaques with XMRV (outlined in Fig. 2). Initially, 1 ml of virus stock was used to inject animals DBL2(d) and DBNP(d), and 1 ml complete medium was injected into animal DBHH(c) as a control. The inoculum titer (determined as $10^{4.5}$ $TCID_{50}$ per ml in LNCaP cells or $10^{5.5}$ $TCID_{50}$ per ml in Mv1Lu cells) was based upon our previous study in rhesus macaques with a recombinant amphotropic murine leukemia retrovirus that resulted in a productive infection with establishment of long-term persistence (our unpublished data) and with SFV (38). Nested PCR analysis of DNAs prepared from whole blood or PBMCs of animal DBL2(d) at

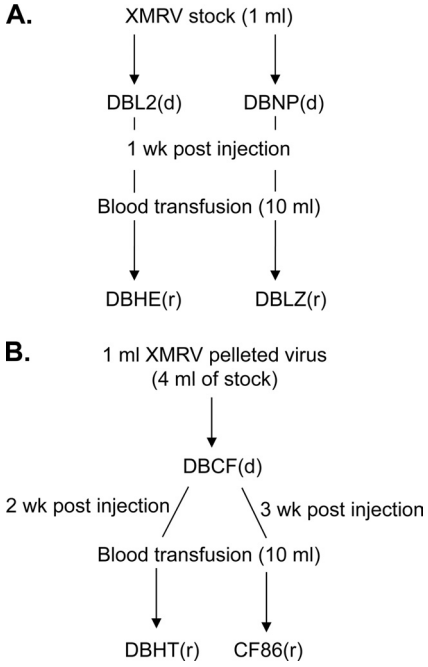

FIG 2 Schematic of monkey injections and blood transfusions. Monkeys were injected with XMRV filtered supernatant virus stock (A) or with pelleted virus resuspended in PBS (pH 7.4) (B).

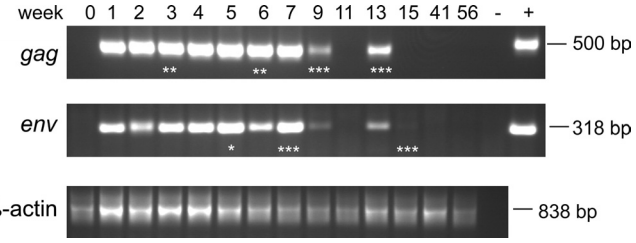

FIG 4 PCR analysis of XMRV infection in animal DBCF(d). Nested *gag* and *env* PCR assays were done on monkey blood (weeks 2 and 3) and PBMC DNAs. Blood from week 0 was collected prior to virus injection. Ten microliters of sample was used for the first PCR with *gag* and *env* primers and for the β-actin PCR assay. The positive (+) control was 10 copies of XMRV cloned DNA spiked in a background of 0.5 μg human cellular DNA. Sizes of the expected PCR-amplified fragments are indicated. Asterisks in the lanes indicate the number of positive PCRs out of 5 replicates (*, 4 out of 5; **, 3 out of 5; ***, 1 out of 5).

weeks 0, 1, 2, 3, 4, 5, 6, 8, and 10 indicated transient detection of XMRV from weeks 2 through 8 using *gag* and/or *env* primers (data not shown). Development of XMRV-specific antibodies against $p30^{CA}$ was seen at week 8 and persisted at 59 weeks. The detection of antibodies against $gp70^{SU}$ was difficult to interpret due to similar-sized bands being detected in the cell lysate; therefore, the results of antibodies developed against Env were based upon p15E (Fig. 3A). PCR analysis of animal DBNP(d) indicated transient detection of XMRV-specific *gag* and/or *env* sequences from weeks 2 through 10 (data not shown), and virus-specific antibodies to Gag and Env were seen at week 6 and persisted at week 59 (Fig. 3B).

In the second study, in an effort to generate a more robust *in vivo* infection, the virus stock was subjected to ultracentrifugation to remove any potential inhibitory or interfering factors in the spent medium, and the resuspended pellet (about 4× concentrated) was injected into a naïve monkey, animal DBCF(d). Western blot analysis indicated detection of Gag ($p30^{CA}$) and Env (p15E) antibodies at week 6 with increasing intensity up to week 41 (last time point tested) (Fig. 3C). Although Western blot analysis is not quantitative, the response seemed to be stronger in animal DBCF(d) than in animal DBL2(d) or DBNP(d), which were injected with the XMRV stock, without prior ultracentrifugation. Nested PCR analysis of DNAs prepared from whole blood or PBMCs indicated amplification of fragments of the expected size with *gag* and *env* primers at weeks 1 to 9 and week 13; a faint band was seen at week 15 with *env* primers (Fig. 4). In some cases when the initial nested PCR results were negative, positive results were seen when samples were tested in an additional four replicates (indicated by asterisks in Fig. 4 and details described in the legend). Negative results were obtained at weeks 11, 15, 41, and 56 in all five replicates.

Evaluation of XMRV transmission by whole-blood transfusion. Initially, based upon previously reported results for the ki-

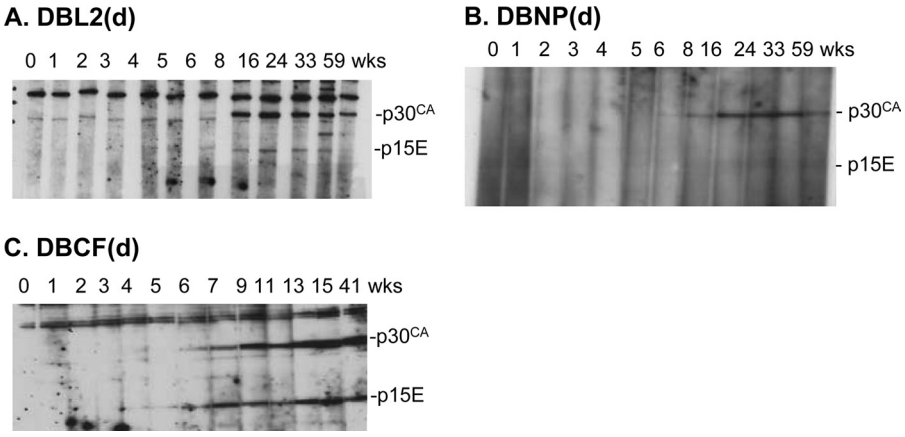

FIG 3 Detection of XMRV-specific antibodies in donor monkeys by Western blotting. Plasma samples, obtained on the day of injection (week 0) and at various weeks following inoculation, were incubated with a nitrocellulose membrane containing lysate prepared from XMRV-infected Mv1Lu cells, and proteins were visualized as described in Materials and Methods. An exposure of chemiluminescence film is shown. The XMRV-specific Gag $p30^{CA}$ and Env p15E proteins are indicated based upon standard markers (MultiMark; Novex, San Diego, CA).

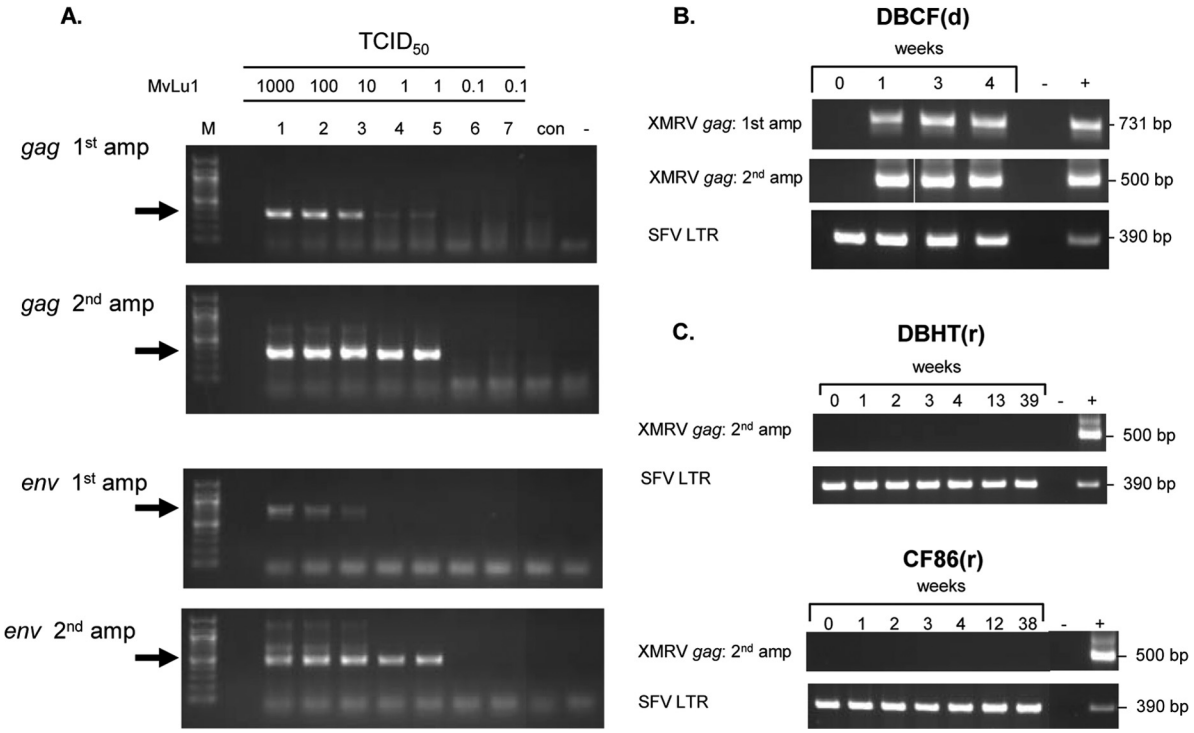

FIG 5 PCR analysis of mink cells cocultured with monkey PBMCs. (A) Serial dilutions of XMRV stock were used to determine the sensitivity of virus detection in Mv1Lu cells. PCR analysis of Mv1Lu cells at day 9 after infection is shown, using *gag* and *env* outer and inner primer sets. Arrows indicate the size of the expected XMRV-specific fragment. Lanes 1 to 7, XMRV $TCID_{50}$ determined in Mv1Lu cells; M, 1-kb marker; con, uninfected-cell DNA; −, no-DNA control for PCR. Monkey PBMCs (5×10^6) were PHA stimulated and cocultured with Mv1Lu cells (400,000 cells), as described in Materials and Methods. (B and C) DNA was prepared from cell pellets obtained at the end of the culture period or upon extensive CPE due to the presence of SFV in the monkey PBMCs, at days 19 to 34 in the case of donor monkey DBCF(d) (B) and at days 16 to 30 and 25 to 30 in the case of blood recipient monkeys DBHT(r) and CF86(r), respectively (C). Results from outer PCR primers or nested PCR analysis for XMRV *gag* and *env* as well as for SFV LTR using outer primers are shown. Expected sizes of PCR-amplified fragments are shown based upon the positive control DNAs (lane +) from XMRV-infected or SFV-infected cells; uninfected DNA is the negative control (lane −). Weeks of isolation of PBMCs used for coculture are indicated. PCR-amplified fragments of SFV at week 0 from animals CF86(r) and DBHT(r) and at week 1 from animal DBCF(d) were sequenced. Additionally, the PCR-amplified fragment of XMRV from week 1 from animal DBCF(d) was sequenced.

netics of XMRV infection in rhesus monkeys (28), whole blood was transfused at week 1 after virus injection from animals DBL2(d) and DBNP(d) into animals DBHE(r) and DBLZ(r), respectively (Fig. 2). Subsequent analysis of monkey PBMC DNAs by nested PCR assays indicated that week 1 donor samples were virus negative, whereas virus was detected transiently from weeks 2 through 8 (data not shown). Additionally, no virus was isolated from PBMCs of animal DBL2(d) 1 week after XMRV injection, based upon nested PCR analysis of DNAs prepared from target cells at day 30 after coculture; mouse β-actin primers amplified the expected 340-bp fragment, indicating the presence of intact DNA in the test samples (data not shown). Similarly, negative results for XMRV isolation were obtained in coculture studies with week 1 PBMCs from animal DBNP(d); however, since the monkey was SFV positive prior to XMRV infection, SFV isolation was demonstrated by using an SFV-specific PCR assay at day 25 after coculture of week 0 and week 1 PBMCs of animal DBNP(d) (data not shown).

Although there was no evidence of XMRV infection in PBMCs of animals DBL2(d) and DBNP(d) at week 1 after virus infection, blood recipient animals DBHE(r) and DBLZ(r) were analyzed for virus transmission in case there was a low level of undetected infection in the PBMCs or virus in the plasma of the donor monkeys. DNAs prepared directly from whole blood or PBMCs at weeks 0, 1, 2, 3, 4, 5, 6, and 9 were negative by nested PCR assays for XMRV *gag* and *env*, and the monkeys were negative for the development of XMRV-specific antibodies from week 0 through week 32 (last time point tested) by Western blotting.

To further evaluate XMRV transmission, blood from animal DBCF(d) (injected with 4× resuspended virus) was transfused at week 2 and week 3 after XMRV injection into animals DBHT(r) and CF86(r), respectively. Subsequent PCR analysis indicated that PBMC DNA from animal DBCF(d) DNA from these time points was positive for XMRV *gag* and *env* (Fig. 4).

The sensitivity of XMRV isolation was evaluated by coculture of PBMCs with Mv1Lu cells followed by detection using PCR assays. Analysis of a 10-fold dilution series of XMRV stock indicated that 1 $TCID_{50}$ could be detected by nested PCR using *gag* and *env* nested primers at day 9 after infection of Mv1Lu cells (Fig. 5A). XMRV-specific *gag* and *env* DNA fragments amplified using nested PCR from infection with 1 $TCID_{50}$ were confirmed by nucleotide sequencing (data not shown).

XMRV was isolated from cocultures at days 19 to 34 using animal DBCF(d) PBMCs from weeks 1, 3, and 4 after XMRV injection. PCR analysis detected *gag* sequences after the first round of PCR; no fragments were amplified from the week 0 or the negative-control sample using nested PCR (Fig. 5B). There were insufficient PBMCs available from week 2 for the coculture assay.

SFV LTR sequences were amplified using outer primers from week 0 in addition to weeks 1, 3, and 4, thus confirming SFV infection in the monkey prior to study initiation. The identity of the XMRV and SFV PCR-amplified fragments was confirmed by nucleotide sequence analysis of the fragments amplified from week 1 PBMC coculture DNA.

Evaluation of animals DBHT(r) and CF86(r), which were transfused with blood from animal DBCF(d), indicated that both blood recipient animals were negative for XMRV infection by nested *gag* and *env* PCR analysis of PBMC DNAs prepared from animals DBHT(r) and CF86(r) at weeks 6, 7, 8, and 9 and at weeks 5, 6, 7, and 8, respectively; additionally, there was no detection of XMRV-specific antibodies up to 32 weeks in the case of animal DBHT(r) and up to 31 weeks in the case of animal CF86(r) (data not shown). To further confirm the absence of XMRV transmission, monkey PBMCs were cocultured with Mv1Lu cells at various times after transfusion. The results of PCR analysis of DNAs prepared from PBMC coculture studies of animal DBHT(r) at days 16 to 30 postcoculture and from animal CF86(r) at days 25 to 30 postcoculture are shown in Fig. 5C: there was no detection of XMRV sequences using nested PCR for *gag* in either monkey. The amplification of SFV LTR sequences from all of the test samples demonstrated the presence of intact DNA in the samples and provided a relevant positive control for virus isolation by coculture of monkey PBMCs. The identity of the SFV fragment amplified from the week 0 sample was confirmed by sequencing. The control samples gave the expected negative and positive results.

Sequence analysis of XMRV infection *in vivo*. XMRV infection in PBMC DNA was seen by nested PCR assays for animal DBCF(d) until week 9 after virus injection and then reappeared at week 13, before becoming undetectable after week 15 with both *gag* and *env* primers. In some cases, a positive result was detected only after testing replicates, which suggested changes in the PBMC viral load during the course of virus infection in the animal. Analyses of XMRV infection in rhesus and pigtailed macaques previously showed that virus replication seems to be restricted due to APOBEC-mediated G-to-A hypermutation (40, 41). Our analysis of PCR-amplified fragments from PBMC DNAs in *gag* for weeks 1, 7, 9, and 13 indicated a few, mostly single-nucleotide changes in the region of *gag* analysis that did not evolve further over time (data not shown). However, analysis of *env* for weeks 5, 6, 7, 9, and 13 indicated significant sequence changes at week 9, which consisted of multiple G-to-A mutations as well as base deletions and insertions. The majority of these changes were also seen at week 13, along with additional mutations and some reversions. The results of the nucleotide sequence analysis indicated that most of the changes seen in *env* were G-to-A mutations and occurred within GG and GA dinucleotides (Fig. 6).

DISCUSSION

The discovery of XMRV as a novel human retrovirus in samples from patients with prostate cancer (1) and chronic fatigue syndrome (2) generated great excitement and debate as well as skepticism (42) regarding the causal relationship of a novel retrovirus with these diseases; however, there were also heightened public health safety concerns related to the lack of knowledge regarding virus transmission and infection in humans. In this study, we demonstrated the lack of XMRV transmission in rhesus macaques that received a whole-blood transfusion from virus-infected donor monkeys during the acute phase of infection. The transfused monkeys tested negative up to 32 weeks after transfusion based upon PCR analysis of PBMC DNA, Western blot analysis, and virus isolation.

XMRV infection was seen in the donor monkeys by direct injection of the virus stock, and the infection seemed enhanced in animal DBCF(d) after injection with a concentrated (4×) virus stock based upon the stronger antibody responses and detection of virus sequences in the PBMCs by PCR assays. This may be due to the removal of spent culture medium that may have contained inhibitory factors or the concentration of virus by ultracentrifugation. Similarly, a robust response was seen previously by Onlamoon et al. upon reinfection of rhesus monkeys using the same inoculum titer after ultracentrifugation through sucrose (28). Regardless, XMRV infection in monkeys is restricted, since robust infection could not be established even after using ultracentrifuged virus. APOBEC3-mediated restriction of XMRV replication has been shown in human cell lines (41, 43, 44) and in monkeys (40, 41). Our study also showed an extensive accumulation of G-to-A mutations and other nucleotide changes in PBMC viral DNA from week 9 in animal DBCF(d), thus corroborating the role of APOBEC3 proteins in limiting XMRV infection in monkeys. Interestingly, in contrast to the results reported previously by Zhang et al., where most of the G-to-A mutations occurred in the context of GA and GC dinucleotides (41), the majority of mutations seen in our analysis involved GG and GA dinucleotides, which is characteristic of APOBEC3G activity in human immunodeficiency virus type 1 (HIV-1) mutations in human PBMCs (45, 46) Thus, it is expected that such APOBEC3-mediated hypermutations would reduce the risk of a productive infection in humans in the case of an inadvertent virus infection. The lack of efficient virus replication in monkeys may also contribute to the lack of XMRV transmission by blood transfusion. Although neutralizing antibodies can contribute to the lack of retrovirus infection (47, 48) and transmission (38), and they were shown to be present as early as week 2 in a pigtailed macaque study after XMRV injection (40), these were not likely to be involved in the lack of XMRV transmission in this study, since similar results were obtained by blood transfusions at week 1, week 2, and week 3.

The results of XMRV studies in macaques seem similar to those with a replication-competent, recombinant amphotropic murine retrovirus generated from a Moloney MLV (MoMLV)-based gene therapy packaging cell line, where large amounts of virus established only transient infection and were "cleared" from the peripheral blood after intravenous injection in normal, immunocompetent animals or a moderately immune-suppressed monkey (49, 50). However, injection of highly immunosuppressed monkeys with a similar recombinant murine retrovirus containing amphotropic *env* sequences resulted in high retrovirus replication and T cell lymphomas in 3 animals after retrovirus-mediated gene transfer (39). XMRV injection showed increasing virus-specific antibody responses in donor monkeys to about 39 weeks, indicating virus replication *in vivo*, whereas in previous studies, responses declined after 16 weeks (28, 40). Although no clinical signs were seen at about 2 years postinfection based upon physical examination, blood chemistry, and complete blood count (CBC) differential, consistent with data from other macaque studies (28, 40), the generation of leukemias with a murine retrovirus under immunosuppressed conditions in monkeys emphasizes the need for caution regarding XMRV exposure in humans.

Previous studies have shown that MLV-related sequences were

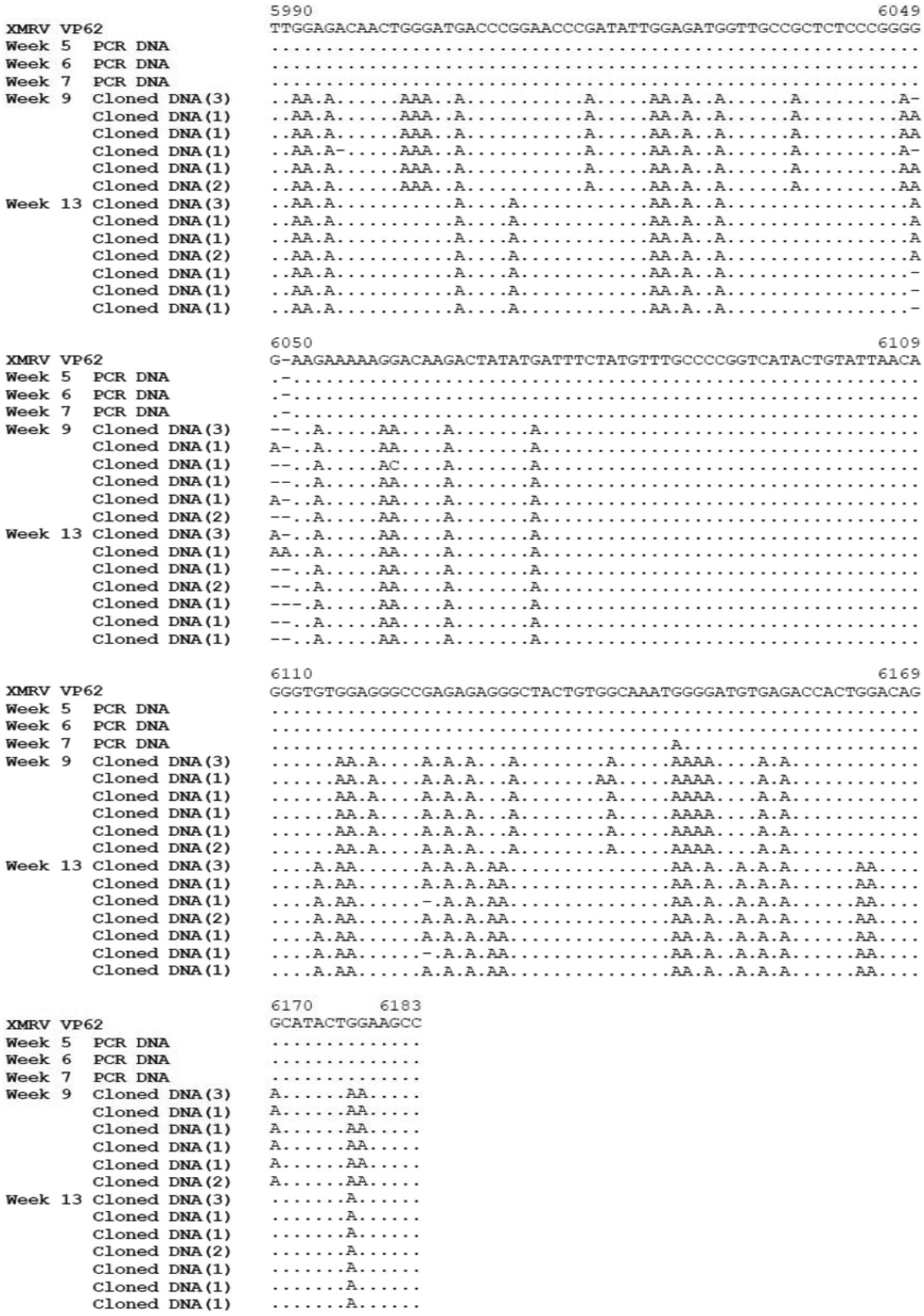

FIG 6 Nucleotide sequence analysis of XMRV in PBMC DNA from animal DBCF(d). Nucleotide sequences in *env* were obtained directly from PCR-amplified fragments in the case of weeks 5, 6, and 7 or after DNA cloning in the case of weeks 9 and 13. Sequences were compared with the analogous region in XMRV VP62; identical bases are indicated by a dot, different bases are shown, and absent nucleotides are indicated by a dash. The nucleotide base numbers are indicated based upon the XMRV VP62 genome (GenBank accession no. EF185282). The numbers in parentheses indicate the number of identical cloned DNAs.

a contaminant in some widely used laboratory reagents (14–16, 19, 51) and also in the research samples that were used for the initial discovery of XMRV (1). The biological properties of XMRV indicate a broad host range and cell tropism, including human cell lines (26, 27, 52), and XMRV contamination was found in several cell lines used for research (52). These results emphasize the need to evaluate XMRV and other MLV contaminations in cell lines used in the laboratory and in the manufacture of biological products (32) as well as to minimize virus exposure to reduce the potential risk of human infection.

XMRV was discovered in prostate cancer tissue using a Virochip DNA microarray (1). Broad nucleic acid-based virus detection technologies such as virus microarrays, massively parallel sequencing (MPS), and long-range PCR with mass spectrometry have recently been used for novel virus discovery. The XMRV story demonstrates the potential use of emerging nucleic acid-based technologies as a powerful tool for evaluation of virus contamination in clinical and biological materials; however, the intensive follow-up highlights the efforts and resources that may be needed to assess the biological relevance and significance of the initial detection of a nucleic acid signal in assays that are based on these technologies. Additionally, the detection of MLV-related sequences as a broad contaminant in research reagents demonstrates the importance of confirming results to determine the origin of a signal using nucleic acid detection assays and to make efforts to use clean starting materials for an accurate interpretation of results. This is further highlighted by our findings that XMRV primer sequences were found in various cell lines, including human cell lines, which resulted in the generation of unexpected fragments in PCR analyses of various cell lines used for research or related to the development of biologicals (32). Thus, although XMRV is not a human retrovirus, it is a "virus of interest" that needs to be considered a potential source of broad contamination with a possible risk of human infections.

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The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy. Use of trade names is for identification only and does not imply endorsement by the U.S. Department of Health and Human Services, the Public Health Service, the Centers for Disease Control and Prevention, or the U.S. Food and Drug Administration.

ADDENDUM IN PROOF

PCR analysis of DNAs prepared from whole blood of all of the animals was found to be negative by testing five replicates of each monkey at about 2.5 years from animals DBL2(d), DBNP(d), DBHE(r), and DBLZ(r) and at about 2 years from animals DBCF(d), DBHT(r), and CF86(r).

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