PBST+5%). The strips were then incubated with 1:100 dilution in PBST+5 percent of plasma or serum, except in case of monkey D1 where 1:500 dilution of plasma was used. The membrane strips were initially incubated for 2 hours at room temperature and then overnight at 4°C on a rocker. The strips were brought to room temperature and washed three times for 5 minutes each in PBST+5 percent and then incubated for 2 hours at room temperature with a 1:500 dilution in PBST+5 percent of horseradish peroxidase (HRP)-conjugated goat anti-monkey IgG (Cappel-ICN Pharmaceuticals Inc., Aurora, OH). The strips were then washed five times for 5 minutes each in PBST, and the protein bands were visualized by chemiluminescence with a substrate system (Supersignal CL-HRP substrate system, Pierce, Rockford, IL). The substrate was added to the membrane strips for 2 minutes, the strips then blotted with paper (Whatman 3 MM, Maidstone, Kent, England) to remove excess substrate and exposed for various times ranging from 5 seconds to 2 minutes with film (BioMax MR, Kodak, Rochester, NY).

Neutralizing antibody endpoint titers were determined in assays with homologous virus (SFV-D1 with plasma from D1 and SFV-D2 with plasma from D2). MRC-5 cells (ATCC CCL-171; human lung fibroblast) were planted in a 24-well plate with 30,000 cells per well (Passage 25) in Eagle's minimum essential medium (modified) with Earle's salt without L-glutamine (Cellgro, Mediatech, Herndon, VA) containing 10 percent heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mmol per L glutamine, 250 U of penicillin per mL, 250 µg of streptomycin per mL, 1× nonessential amino acids (MEM-NEAA 100×, Quality Biological, Inc., Gaithersburg, MD) 1 mmol/L sodium pyruvate in a total volume of 2 mL. Cells were incubated overnight at 37°C, and 0.2 mL was removed to replace with test sample. Monkey plasma (heat-inactivated) was diluted twofold (ranging initially from 1:50 to 1:1600) in PBS, pH 7.4, without calcium and magnesium (Quality Biological, Inc.), initially ranging from 1:50 to 1:1600. Plasma samples were incubated for 1 hour at room temperature with equal volume of SFV (100 $TCID_{50}$ per 0.1 mL), after which 0.2 mL was removed and added to each well, in triplicate. The tray was incubated at 37°C and cells observed for cytopathic effect (CPE) up to Day 13, when the final results were recorded. The antibody endpoint was the highest dilution of plasma that inhibited CPE in all replicate wells.

DNA preparation and PCR analysis

Cryopreserved PBMNCs were recovered in RPMI and washed with cold PBS (without Ca^{2+} and Mg^{2+}), and DNA was prepared with a DNA blood mini kit according to the manufacturer's protocol (QIAamp, Qiagen, Valencia CA) except that all spins were at 15,800 × g at room temperature and the DNA elution time was increased to 5 minutes at room temperature. DNA was aliquoted and stored at −80°C.

SFV sequences in PBMNC DNA were amplified by PCR with previously described conditions with set B outer primer pair and inner primer pair (3+5 and 6+7, respectively[21]). The sensitivity of the outer primer set was shown to be 10 viral copies in 10^5 cell equivalents of cellular DNA. The identity of the SFV sequences was confirmed by nucleotide sequence analysis of gel-purified DNA fragments (gel DNA recovery kit, Zymoclean, Orange, CA), obtained with primers 6 and 7. PCR primers, which amplified an 838-bp fragment of the human β-actin gene (Clontech, Palo Alto, CA), were used as a control for the presence of DNA in the sample. The PCR mixture without DNA was used as the negative control.

Nucleotide sequence analysis

Nucleotide sequence reactions were set up with primers 6 and 7, according to the protocol with a cycle sequencing kit according the manufacturer's protocol with 5X sequencing buffer (BigDye Terminator Version 3.1 cycle sequencing kit, Applied Biosystems, Foster City, CA). The sequence reactions were purified with spin columns (CentriSep, Princeton Separations, Adelphia, NJ), and sequences were determined with a DNA sequencing system (ABI Prism 377, Perkin-Elmer Applied Biosystems, Foster City, CA).

Blood processing and virus isolation

PBMNC and plasma were prepared from blood containing EDTA as preservative (SeraCare Bioservices, previously BBI Biotech Research Laboratories, Inc., Gaithersburg, MD). Plasma was aliquoted and stored at −80°C. PBMNC were prepared by the Ficoll-Hypaque method, aliquoted, and cryopreserved. For virus isolation, PBMNC were stimulated in a 24-well plate with 5 µg per mL phytohemagglutin (PHA; Murex Biotech Ltd, Dartford, Kent, England) for 72 hours in RPMI containing 10 percent (1000 U) human interleukin-2 (Roche, Indianapolis, IN), 10 percent FBS (heat-inactivated 56°C for 30 min; Hyclone), 2 mmol per L glutamine, 250 U of penicillin per mL, 250 µg of streptomycin per mL. PHA-stimulated PBMNC were added to *M. dunni* cells (1.3×10^6-1.9×10^6) in a 75-cm^2 flask for coculture in Dulbecco's minimum essential medium containing 10 percent FBS, 2 mmol per L glutamine, 250 U of penicillin per mL, 250 µg of streptomycin per mL in a total volume of 20 mL. Cultures were passaged every 3 or 4 days when the cells reached confluency and maintained until culture termination due to extensive CPE or at least 30 days. PBMNC were added back to the cultures for three passages after the initial coculture. Filtered supernatants were collected and stored at various times during the culture period for Mn^{2+}-dependent reverse transcriptase (RT)

assay.[21] SFV identity was confirmed at culture termination by PCR amplification and nucleotide sequence analysis.

PBMNC viral load determination

MRC-5 cells were planted overnight as described above for the neutralization assay. One-milliliter of medium was removed and replaced with 1 mL containing fivefold serially diluted monkey PBMNC ranging from 1×10^6 cells per mL to 320 cells per mL per well. Each dilution was tested in at least four replicates. The plate was incubated at 37°C for 14 days. Filtered supernatant was collected and analyzed for SFV by a PCR-enhanced RT assay (STF-PERT[22]). The $TCID_{50}$ was calculated by the Kärber method[23] and infectious units per million total PBMNC (IUPM) expressed as the reciprocal of the $TCID_{50}$.

RESULTS

SFV infection occurred in two recipient monkeys (R1 and R2) that were transfused with blood from donor animal D1, but not in the two animals (R3 and R4) that received blood from donor animal D2 or in a saline-injected control animal.

Detection of SFV-specific antibodies in transfused monkeys

Plasma from study animals was analyzed for SFV-specific antibodies at various times after transfusion. The results of dot blot assays are shown in Table 1. The earliest time at which SFV antibody was detected in R1 and R2 was 22 and 16 weeks, respectively, after which time both animals remained positive. The control animal was negative at all tested times.

The antibody status of the animals was further evaluated by Western blot analysis. The results in Fig. 1 indicate the presence of SFV antibodies as early as Week 1, which decreased over time, representing passive transfer of donor antibodies. The resurgence of antibodies was seen at Week 22 in R1 and at Week 16 in R2 indicating the development of antibodies in response to virus infection after transfusion. Antibodies to SFV proteins persisted at Week 48, the last time point tested: the 65K and 70K proteins most likely correspond to the diagnostic Gag doublet seen in all infected species (p68/71[24]). Passive antibody transfer also occurred in R3 and R4 after blood transfusion from D2; however, there was no evidence of new antibody development due to virus infection (data not shown). No SFV-specific antibodies were seen in the control animal.

Detection of SFV sequences in monkey PBMNC

The kinetics of SFV infection by blood transfer were evaluated by PCR analysis of monkey PBMNC DNA. SFV-specific primers amplified a 349-bp fragment from R1 and R2 from PBMNC at Week 8 after transfusion and thereafter (Fig. 2). The expected size β-actin fragment was seen in all the samples, indicating the presence of intact DNA in the samples. The identity of the PCR-amplified fragment from

TABLE 1. Development of SFV-specific antibodies by blood transfusion*

Weeks after transfusion	Monkeys		
	R1	R2	Control
0	–	–	–
1	–	–	–
2	–	–	–
4	–	–	–
8	–	–	–
11	–	–	–
16	–	+/–	–
22	+	+	–
30	+	+	–

* All samples were run in the same assay, and each sample was analyzed in two independent assays. Differences in the results in the two assays are indicated. Negative is less than 1:5.

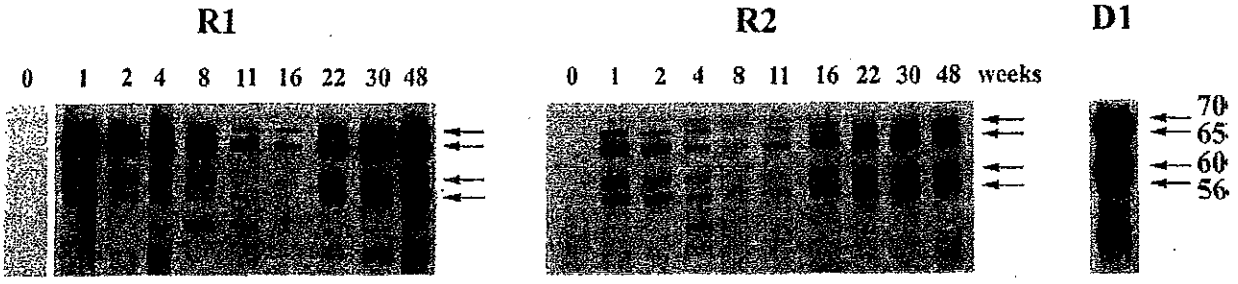

Fig. 1. Detection of SFV-specific antibodies by Western blot analysis. Monkey plasma samples, obtained on day of blood transfusion (Week 0) and at various weeks after transfusion (except Week 16, where serum was used), were incubated with immunoblot strips containing lysate prepared from SFV-2-infected *M. dunni* cells and proteins visualized as described under Materials and methods. A 5-second exposure of the autoradiogram is shown. The molecular masses of prominently visible, SFV-specific proteins, calculated from standard markers (MultiMark, Novex, San Diego, CA), are indicated in kilodaltons.

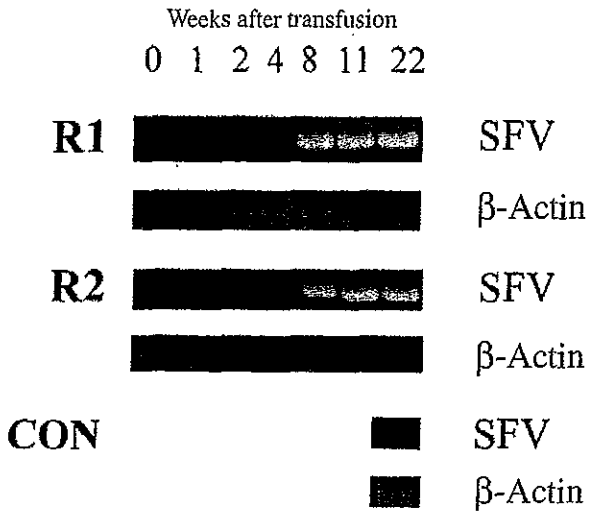

Fig. 2. Detection of SFV in monkey PBMNCs by PCR assay. SFV-specific primers were used to analyze PBMNC DNA as described under Materials and methods. DNA samples were prepared from PBMNCs that were obtained on the day of blood transfusion (Week 0) and at the indicated times after transfusion. PCR amplification with β-actin primers confirmed presence of DNA in the samples. CON = control.

the 22-week sample of R1 and R2 was confirmed by nucleotide sequence analysis. As shown in Fig. 3, the SFV sequences in R1 and R2 were identical to the SFV in D1. SFV-specific sequences were not detected in R3 and R4 at any time up to 30 weeks (the last tested time), including early time after transfusion, where passive antibodies were present (data not shown). The control animal was negative by PCR with SFV primers.

SFV isolation from monkey PBMNC

To determine whether the SFV sequences detected in R1 and R2 were associated with an infectious virus, monkey PBMNCs from Week 11 and Week 22 after transfusion were cocultured with *M. dunni* cells. The cultures were monitored for replicating SFV by the appearance of CPE in the cell monolayer and by RT production in cell-free supernatant. The RT results, shown in Fig. 4, indicate earlier virus isolation with the Week 11 sample from both R1 and R2, with culture termination due to extensive CPE at Day 14: in the case of the Week 22 sample, there was a slightly delayed kinetics of virus isolation with culture termination on Day 16. This difference in the kinetics of virus isolation was also evidenced by CPE detection in the cocultures, which was seen on Day 9 in the case of the Week 11 sample and on Day 11 with the Week 22 sample. The kinetics of virus isolation with PBMNC from the day of blood transfusion for D1 showed that CPE was seen on Day 11 with culture termination on Day 18. No virus was detected in PBMNC from R1 and R2 on the day of transfusion nor at any time from the control animal. There was no evidence of virus isolation from PBMNC of R3 and R4 at any time point tested including 1 year after blood transfusion, the last tested time; virus was isolated from D2 on the day of blood transfer (data not shown).

The identity of the viruses isolated in the coculture experiments with the Week 11 sample from R1 and R2 was confirmed by PCR amplification and nucleotide sequence analysis: the results indicated sequence identity with SFV-D1 (data not shown).

Characterization of donor monkeys

The selection of D1 and D2 as donors was initially based on the results of earlier infectivity studies, which demonstrated that the SFVs isolated from the PBMNC of D1 and D2 were distinct in their replication kinetics and CPE development: SFV-D1 had high replication and rapid CPE as compared with SFV-D2 (data not shown). To further investigate the differences in SFV transmission by blood transfusion with D1 and D2, the neutralizing antibody titer and PBMNC viral load were determined on stored samples from the animals. The results indicated a neutralizing antibody endpoint titer of 1:50 for D1 and 1:800 for D2. PBMNC viral load analysis indicated 32.4 IUPM for D1 and 3.8 IUPM for D2. Additionally, a retrospective analysis the CBC differential count indicated that the WBC count in D2 was about half of that in D1.

DISCUSSION

The identification of SFV-seropositive blood donors has raised safety concerns regarding SFV transmission by blood transfusion. A study analyzing recipients of blood components such as RBCs, filtered RBCs (WBC-reduced), PLTs, and fractionated plasma from one SFV-infected donor demonstrated absence of virus transmission,[16] however, PBMNC, which are known targets of SFV infection, were not examined and the results are limited by the sample size. Based on a theoretical risk the CDC has been counseling infected people not to donate blood.[16] To evaluate the potential risk of SFV transmission by blood and blood products, we have initially determined virus transmission by whole-blood transfusion in a monkey model. Blood was transferred from two donor animals that were naturally infected with SFVs that had distinct replication kinetics and nucleotide sequences. Interestingly, SFV transmission only occurred with D1: antibodies developed at 16 to 22 weeks and persisted approximately 1 year after transfusion (the last time tested); SFV sequences were detected by PCR at 8 weeks after transfusion, and infectious virus was isolated from PBMNC at Week 11 and Week 22. The lack of virus transmission with blood transfusion from D2 was unexpected because SFV has an

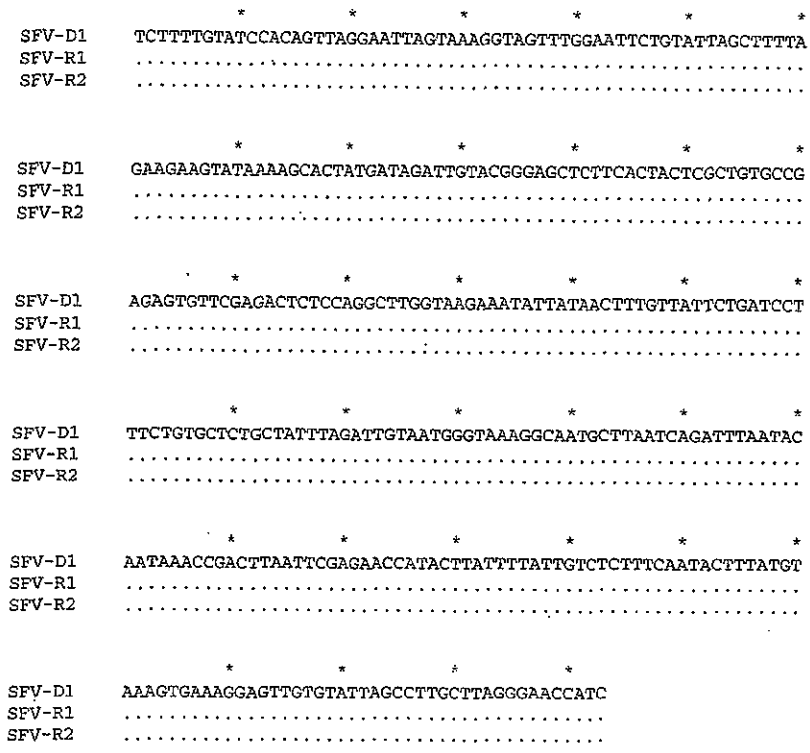

Fig. 3. Nucleotide sequence identification of SFV sequences in blood recipient monkeys. Nucleotide sequences of SFV in R1 and R2 (designated as SFV-R1 and SFV-R2, respectively) were determined from DNA fragments that were PCR-amplified from PBMNC at 22 weeks after transfusion (shown inFig. 2). Sequence comparison with SFV in the donor animal (SFV-D1) are shown: dots indicate base identity; asterisks indicate base count.

exceptionally broad host range and tissue tropism and is easily transmitted in NHPs, albeit via the saliva.[3] Different factors may contribute to retrovirus transmission such as virus load in the inoculum and fitness of the donor virus. Additionally, neutralizing antibodies have been shown to block SHIV infection of macaques.[25,26] Antibody analysis of D1 and D2 indicated a significantly higher neutralizing antibody endpoint titer in D2 compared to D1 (1:800 versus 1:50, respectively) suggesting that neutralizing antibodies may play a role in SFV transmission. Studies are under way to investigate the contribution of antibody titer in the failure of SFV transmission by D2. The results of these studies may provide insight regarding factors involved in SFV transmission and in assessing the risk of virus transmission by blood donors.

High viral load is an important determinant of virus transmission in HIV-1 infection.[27] In the case of SFV infection, the virus largely infects lymphocytes and monocytes,[3,14,15] and it is believed that virus is mostly cell-associated with no detectable virus in the plasma. Therefore, we initially determined the PBMNC viral load of D1 and D2: the results indicated that the IUPM was 32.4 and 3.8, respectively. Interestingly, this is similar to the PBMNC viral load reported in chronic infection with SIV in African green monkeys[28] and HIV-1 in humans.[29] Although the blood transfer volume was the same (10 mL), based on the CBC differential, it was found that D1 had twice the number of WBC as D2: thus approximately 29×10^6 PBMNC were transfused in case of D1 and 15×10^6 in case of D2 so that the approximate number of infected cells transferred by D1 was 940 cells and 57 by D2. Additional studies will be performed to determine whether the PBMNC viral load represents the total number of infected cells in blood and the contribution of plasma viral load, if any, in SFV transfusion transmission. It should be noted that virus fitness[30] may play an important role in virus transmission from D1 based upon in vitro studies indicating that SFV-D1 had earlier replication kinetics and more rapid CPE development than SFV-D2 (data not shown). The relationship between virus fitness and SFV transmission will be investigated to assess the risk of infection by blood transfusion.

Interestingly, virus isolation occurred with more rapid kinetics with the Week 11 PBMNC samples from R1 and R2 than with the Week 22 samples (Fig. 4). Furthermore, the kinetics of virus isolation from PBMC of chronically infected D1 was similar to that of Week 22 samples. This result suggests a higher PBMNC viral load early after infection, with a subsequent lower set point in long-term infection. To evaluate the kinetics of virus infection in vivo, longitudinal analysis of PBMNC viral load will be done on stored samples, including quantitative analysis by TaqMan PCR. Additionally, corresponding plasma samples will be tested for evidence of any SFV viremia. Analysis of PBMNC and plasma viral load may identify a high-risk window period of SFV transmission by blood transfusion. It is noteworthy that the apparent reduction in viral load in the Week 22 PBMNC samples coincided with the increase in SFV-specific antibodies (Table 1 and Fig. 1), thereby suggesting a potential role of neutralizing antibodies in reducing virus replication.

The consequences of cross-species transmission of retroviruses are unpredictable and may not be noticed for an extended period until there is a clinical outcome. This is most effectively evidenced by HIV-1, which was discovered in 1983 due to the AIDS epidemic[31] more than 50 years after the initial cross-species infection with SIV.[32,33] The lack of disease associated with SFV in any spe-

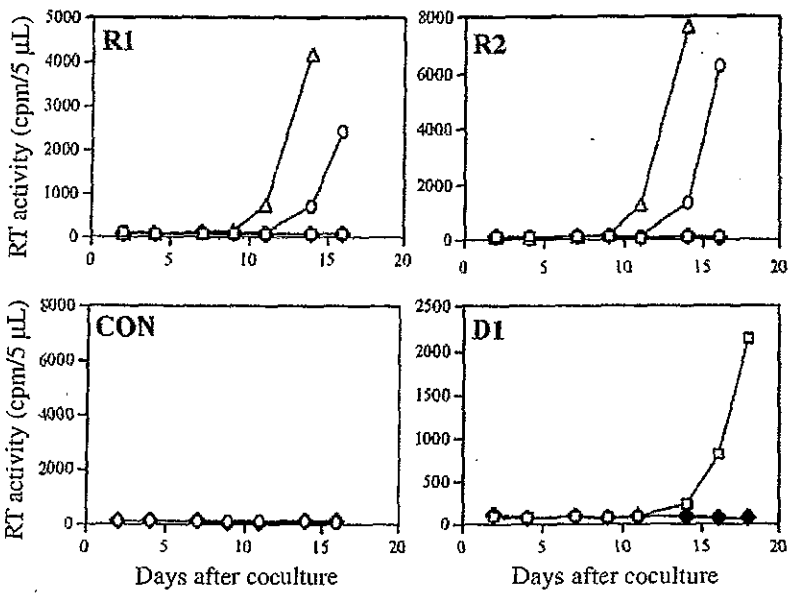

Fig. 4. SFV isolation from monkey PBMNC. PBMNC of R1 and R2, obtained on the day of blood transfer and at Weeks 11 and 22 after transfusion (2.0×10^6-2.3×10^6) were PHA-stimulated and cocultured with *M. dunni* cells, until the cultures were terminated due to extensive CPE. PBMNC from the control animal (CON: Week 22; 2.4×10^6) and donor D1 (day of blood transfusion; $<2.0 \times 10^6$) were PHA-stimulated, and cocultures set up as controls for PBMNC from negative and positive monkey, respectively. *M. dunni* cells without monkey PBMNC were included as cell culture control. Filtered supernatant was collected during the culture period and assayed for RT activity (mean ± standard deviation was calculated from two spots). Day of blood transfusion, □; Week 11 after transfusion, △ Week 22 after transfusion, ○; *M. dunni* control, ◆.

cies is an enigma,[3,4] especially since foamy viruses can be highly cytopathic in cells in vitro.[4] Due to the stable integration and long-term persistence of infectious viral sequences in the host genome, SFV might have an unexpected clinical outcome. Thus, similar to other retroviruses of public health impact, it is prudent take appropriate measures to avoid SFV exposure and infection.

The absence of known disease and lack of transmission in humans does not negate health concerns related to SFV infection in humans due to insufficient data. Demonstration of SFV infection by blood transfusion in a monkey model indicates potential risk for virus transmission in humans. The results support consideration of appropriate safeguards against exposure to SFV, or any other simian agent, through the human blood supply.

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識別番号・報告回数		回	報告日 年　月　日	第一報入手日 2006 年 4 月 27 日	新医薬品等の区分 該当なし	総合機構処理欄
一般的名称			研究報告の公表状況	Development of an improved method of detection of infectious parvovirus B19 Wong, S. and Brown, K.E. J. Clin. Virol., 35, 407-413 (2006)	公表国 米国	
販売名（企業名）						

研究報告の概要

パルボウイルスB19は，ヒトに対してのみ病原性をもつパルボウイルスであるが，急性感染後は赤血球前駆細胞で高濃度まで複製する。その時点で多くの場合は無症候であるので，高度に汚染した血液であっても血液供給される。その上，非エンベロープであるパルボウイルスB19は現在の方法では容易に不活性化されない。この研究では様々な細胞株のB19感染に対する感受性を比較し，中和抗体と同様にウイルス感染を検出する様々な方法を評価した。
UT7/Epo-S1細胞株は，B19感染に最も高い感受性があることが判明した。そしてこの株では，間接免疫蛍光法により容易にB19カプシドタンパクが染色された。最も高感度の感染症分析はRNA転写を検出するRT-PCRあるいは定量的RT-PCR法であった。また，RNA転写を検出するハイスループット分析法が開発され，血漿プールに含まれた感染ウイルスを高力価で検出できた。さらに本分析法を確認する手段として血清で中和抗体を検出した。その結果，抗B19抗体を含む血清でプレインキュベートしたB19ウイルス感染UT7/Epo-S1細胞株では，RNA転写が顕著に減少していた。ここで紹介された分析法は，現存するものより多くの利点を示している。

使用上の注意記載状況・その他参考事項等

BYL-2006-0225

報告企業の意見	今後の対応
弊社血漿分画製剤の製造工程における各段階におけるウイルス除去効果は，パルボウイルス B19 のモデルとしてブタパルボウイルスを用いて確認されている。ヒトトランスフェリンでは 5.9log 以上，ヒトアルブミンでは 6.8log 以上，またヒト免疫グロブリンでは 9.3log までウイルス除去が可能である。したがって，弊社の血漿分画製剤では，ウイルス伝播のリスクは極めて低い。	現時点で新たな安全対策上の措置を講じる必要は無いと考える。引き続き関連情報の収集に努める。

(12)

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Development of an improved method of detection of infectious parvovirus B19

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Abstract

Background: Parvovirus B19, the only known pathogenic human parvovirus is the aetiologic agent of erythema infectiosum, transient aplastic crisis, pure red cell aplasia, and hydrops fetalis. Transmission is either by respiratory secretions or, as it can be present at high titre in plasma, by blood and blood products. B19 is only cultured with difficulty in vitro, and there is no readily available assay for detecting B19 infectivity or neutralizing antibodies.

Objectives: In this study, we evaluated different methods to detect viral infection for the purpose of developing automated methods for large-scale testing of viral infectivity, development of neutralizing antibody and viral inactivation assays.

Study design: Different cell lines were evaluated for their ability to support B19 infection and assays tested for sensitivity and ease of performing. A high-throughput assay was validated by determining infectious virus in blood pools and for determining neutralizing antibody in sera.

Results: B19 protein production was detected by immunofluorescence (IF) staining and increased viral DNA production by dot blot hybridization and quantitative PCR. The detection of RNA transcripts by RT-PCR assay and quantitative RT-PCR (qRT-PCR) was used as an indirect marker for infection. Of the cell lines tested, the subclone UT7/Epo-S1 showed the greatest sensitivity to B19 infection, with detection of viral transcripts by qRT-PCR the preferred assay. The assays were validated by experiments to determine the infectious titre of sera from acutely infected humans, to evaluate the presence of infectious virus in human donor plasma pools and to measure neutralizing antibodies.

Keywords: Parvovirus B19; Erythrovirus infections; Neutralizing antibody

1. Introduction

Parvovirus B19 is the only known parvovirus pathogenic to humans. It is associated with a number of diseases including erythema infectiosum ("fifth disease") in children, arthropathy commonly seen in women, transient aplastic crisis in individuals with high red cell turnover, pure red cell aplasia in immunocompromised patients, and hydrops fetalis following infection during pregnancy (Young and Brown, 2004).

B19 is highly erythrotropic and replicates to high titre in erythroid progenitor cells. In healthy individuals, at the height of the transient viraemia, viral titres as high as 10^{13} genome equivalents (ge)/mL are detectable. Individuals are often asymptomatic at this time, and highly viraemic blood donations do enter the blood supply. In addition, as the virus has only a small (5600 nucleotide) DNA genome, and is non-enveloped, the virus is relatively heat resistant (Schwarz et al., 1992) and not removed by solvent/detergent methods normally used to inactivate virus (Mortimer et al., 1983b; Sayers, 1994). Depending on the sensitivity of the assay, B19 DNA can be detected in between 0.11% and 0.003% of blood donor samples (Jordan et al., 1998; McOmish et al., 1993; Mortimer et al., 1983b; Tsujimura et al., 1995; Wakamatsu et al., 1999), and transmission of B19 infection to recipients of both blood

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