Inactivation of viruses in platelet concentrates by photochemical treatment with amotosalen and long-wavelength ultraviolet light

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BACKGROUND: Viral contamination of platelet (PLT) concentrates can result in transfusion-transmitted diseases. A photochemical treatment (PCT) process with amotosalen-HCl and long-wavelength ultraviolet light (UVA), which cross-links nucleic acids, was developed to inactivate viruses and other pathogens in PLT concentrates.

STUDY DESIGN AND METHODS: High titers of pathogenic or blood-borne viruses, representing 10 different families, were added to single-donor PLT concentrates containing 3.0×10^{11} to 6.0×10^{11} PLTs in approximately 300 mL of 35 percent plasma and 65 percent PLT additive solution (InterSol). After PCT with 150 μmol per L amotosalen and 3 J per cm^2 UVA, residual viral infectivity was assayed by sensitive cell culture or animal systems.

RESULTS: Enveloped viruses were uniformly sensitive to inactivation by PCT whereas nonenveloped viruses demonstrated variable inactivation. Log reduction of enveloped viruses for cell-free HIV-1 was >6.2; for cell-associated HIV-1, >6.1; for clinical isolate HIV-1, >3.4; for clinical isolate HIV-2, >2.5; for HBV, >5.5; for HCV, >4.5; for DHBV, >6.2; for BVDV, >6.0; for HTLV-I, 4.2; for HTLV-II, 4.6; for CMV, >5.9; for WNV, >5.5; for SARS-HCoV, >5.8; and for vaccinia virus, >4.7. Log reduction of nonenveloped viruses for human adenovirus 5 was >5.2; for parvovirus B19, 3.5->5.0; for bluetongue virus, 5.6-5.9; for feline conjunctivitis virus, 1.7-2.4; and for simian adenovirus 15, 0.7-2.3.

CONCLUSION: PCT inactivates a broad spectrum of pathogenic, blood-borne viruses. Inactivation of viruses in PLT concentrates with amotosalen and UVA offers the potential to prospectively prevent the majority of PLT transfusion-associated viral diseases.

Platelet (PLT) transfusions may result in the transmission of viral and bacterial diseases and cause adverse immune reactions. The advent of specific and sensitive tests for viral contamination has greatly reduced transfusion-associated transmission of selected viral diseases.[1] Nevertheless, multilayered testing has not eliminated all viral contamination because most

ABBREVIATIONS: CID_{50} = chimpanzee infectious dose; DHBV = duck hepatitis B virus; FFU = focus-forming units; MP(s) = minipool(s); PCT = photochemical treatment; PFU = plaque-forming units; PPV = porcine parvovirus; SARS-HCoV = severe acute respiratory syndrome-human coronavirus; SDP = single-donor PLT concentrates; TCID = tissue culture infectious dose; UVA = long-wavelength ultraviolet light; WNV = West Nile virus.

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tests are insensitive during the window period before seroconversion of an infected donor.[2] Collection of blood during the window period is likely the most important source of residual human immunodeficiency virus (HIV) infections.[3] Nucleic acid testing (NAT) of minipools (MPs) has reduced, although not eliminated, the window period for newly infected donors, but may not detect those with low HIV viral burdens during the acute phase.[4] Furthermore, some potential donors with silent chronic viral infections, such as hepatitis B virus (HBV), may have very low viral burdens without detectable antiviral antibodies and thus remain undetected by MP NAT.[5] With a combination of serologic and MP NAT in place, the residual risk of contamination for selected viruses has decreased to less than 1:2,000,000 for HIV-1 and -2 and human T-lymphotropic virus (HTLV)-I and -II, less than 1:1,000,000 for hepatitis C virus (HCV), and less than 1:200,000 for HBV.[1,2]

Despite improving the safety of blood components, testing remains a reactive approach to blood safety. The contaminating organisms must be identified before sensitive tests can be developed. New pathogens, such as West Nile virus (WNV), continue to enter the donor population and may be transmitted before a sensitive test is in place.[6] The recent emergence of new strains of pathogenic viruses, such as severe acute respiratory syndrome-human coronavirus (SARS-HCoV), demonstrates once again the susceptibility of the world's blood supply to previously unknown viruses that can spread globally in a short amount of time.[7,8] In addition, cytomegalovirus (CMV) exposure is a significant risk for immunocompromised patients receiving blood component therapy; however, testing and leukoreduction may fail to reach a zero-risk level for prevalent pathogens such as CMV.[9,10]

In contrast, pathogen inactivation technologies offer a proactive approach and the potential to further improve blood safety. To reduce the risks associated with PLT transfusions, a photochemical treatment (PCT) process utilizing a combination of the psoralen amotosalen-HCl and long-wavelength ultraviolet light (UVA) has been developed and introduced into clinical practice in Europe.[11] In vitro studies and extensive clinical testing have demonstrated that PCT inactivates a variety of pathogens while preserving the hemostatic properties of PLTs.[12-18] A recent report provided a summary of the broad-spectrum bacterial inactivation data in PLTs with the PCT process.[19] This report summarizes the viral inactivation data obtained to date.

MATERIALS AND METHODS

PLT concentrates

Early experiments were conducted with single-donor PLT concentrates (SDP) collected with a blood cell separator (CS3000, Baxter Healthcare, Deerfield, IL) with an A35 collection chamber. Later experiments were conducted with SDP collected with another cell separator (Amicus, Baxter Healthcare). Each SDP contained 3.0×10^{11} to 6.0×10^{11} PLTs suspended in approximately 300 mL of 35 percent plasma and 65 percent PLT additive solution (InterSol, Baxter Healthcare) in a 1-L plastic container (PL-2410, Baxter Healthcare).

PCT and UVA illumination devices

PCT was performed with amotosalen-HCl (150 µmol/L) and UVA (320-400 nm). The processing device utilizes a closed system consisting of a series of plastic containers and illumination source (Fig. 1). Early experiments were conducted with a prototype UVA illumination device capable of processing one PLT unit at a time (Model 4R4440, Baxter Healthcare). The output of this device was approximately 15 to 20 mW per cm^2 permitting delivery of a 3 J per cm^2 UVA treatment dose in approximately 3 to

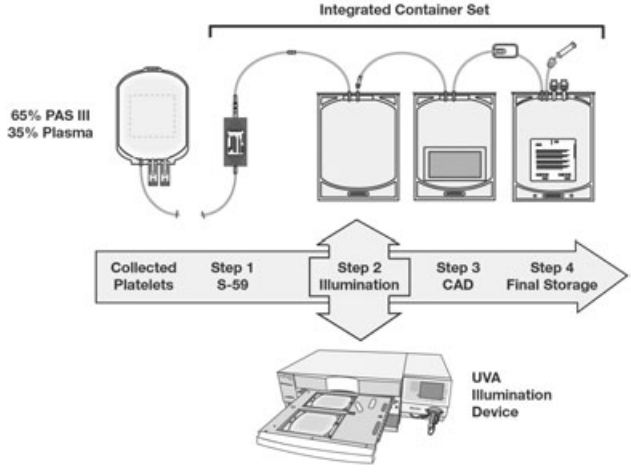

Fig. 1. PCT system. The PCT system consists of an illumination device and a series of plastic containers. The disposable set provides a single-use, closed, integrated system for pathogen inactivation treatment of a PLT product (300 mL). The PLT product in a mixture of 35 percent plasma and 65 percent InterSol (PAS III) is connected via a sterile connection device and passed through a container of amotosalen-HCl (also known as S-59) into an illumination container (Step 1). The integrated set is placed in the UVA illumination device that provides a 3 J per cm^2 dose of UVA to the mixture (Step 2). After illumination for 3 to 6 minutes, the PLTs are transferred to a second container for treatment with a compound adsorption device (CAD) to lower the levels of residual amotosalen and free photoproducts (Step 3). After a minimum of 4 hours of CAD treatment, the PCT PLTs are transferred to a PL-2410 plastic container for storage up to 5 days (Step 4). The CAD step was not used in the viral inactivation experiments described in this report.

4 minutes. Later experiments used a microprocessor-controlled UV illumination system capable of processing 2 PLT units at a time (Model FTX 1116, Baxter Healthcare). The UV illumination system delivers a 3 J per cm^2 UVA treatment dose to each PLT unit in approximately 3 to 6 minutes. Both illumination devices use F15T12-BL fluorescent lamps mounted above and below the illumination tray and are air-cooled for temperature control. The 3 J per cm^2 UVA treatment doses delivered by the two illuminators are equivalent as measured by photodegradation of amotosalen. PLT concentrates were illuminated in both devices while being agitated with reciprocal shaking.

General design of inactivation experiments

The viruses evaluated in this report represent the most relevant blood-borne viruses in transfusion medicine, including all viruses routinely screened for and model viruses representing a spectrum of enveloped and nonenveloped viruses (Table 1). The guidelines issued by CPMP and the FDA were considered in selecting viruses of various genome type and/or size and resistance to inactivation.[20,21] Ten different virus families were represented: retroviruses (HIV-1, HIV-2, HTLV-I, HTLV-II), hepadnaviruses (HBV, duck hepatitis B virus [DHBV]), flaviviruses (HCV, bovine viral diarrhea virus [BVDV], WNV), herpesvirus (CMV), coronavirus (SARS-HCoV), poxvirus (vaccinia virus), adenoviruses (human adenovirus 5, simian adenovirus 15), parvovirus (B19, porcine parvovirus [PPV]), reovirus (bluetongue virus), and calicivirus (feline conjunctivitis virus).

For each experiment, a full-sized SDP unit (300 mL, unless otherwise specified) was spiked with approximately 10^6 infectious units of a virus per milliliter of PLT concentrate. For some viruses, by use of the highest possible achievable titer resulted in spikes of lower infectious units per milliliter. An aliquot was drawn for determination of the pretreatment viral infectivity titer. The spiked PLT mixture was treated with 150 µmol per L amotosalen and 3 J per cm^2 UVA. After treatment, a sample was withdrawn for measurement of the posttreatment viral infectivity titer. Four replicate experiments (unless otherwise specified) with four independent units of SDP were performed for each virus. The infectious viral titer was measured with cell culture methods or animal systems. In the cell culture assay, most PLT samples were first diluted with tissue culture medium and incubated at approximately 37°C for at least 1 hour to allow time for clot formation, which was then removed. In some experiments, as indicated, heparin sulfate (15 U/mL) was added to prevent clotting. The clot-free supernatant was used for viral assay. The inoculum and infectivity assay for each virus are briefly described below.

Cell-free and cell-associated HIV-1

Both cell-free and cell-associated HIV-1, strain IIIB, were evaluated.[22] Cell-associated HIV-1 was produced by harvesting chronically infected H9 cells (gift from C.V. Hanson) and adding these directly to the PLT unit. Cell-free HIV-1 was produced by vigorous vortexing of the infected H9 cells to release virus from the cell surface. Cellular

TABLE 1. Pathogenic and blood-borne viruses selected for inactivation studies

Virus	Routinely screened	Genome*	Envelope	Size (nm)	Resistance to physicochemical reagents†
HIV-1	Yes	ssRNA	Yes	80-130	Low
HIV-2	Yes	ssRNA	Yes	80-130	Low
HTLV-I	Yes	ssRNA	Yes	80-130	Low
HTLV-II	Yes	ssRNA	Yes	80-130	Low
HBV	Yes	ss/dsDNA	Yes	40	Medium
DHBV (a model for HBV)	No	ss/dsDNA	Yes	40	Medium
HCV	Yes	ssRNA	Yes	40-50	Medium
BVDV (a model for HCV)	No	ssRNA	Yes	40-50	Medium
WNV	NAT (IND)‡	ssRNA	Yes	40-50	Medium
CMV	On demand	dsDNA	Yes	150-200	Low-medium
SARS-HCoV	No	ssRNA	Yes	100-120	Medium
Vaccinia	No	dsDNA	Yes	270-350	Medium
B19	No	ssDNA	No	18-26	Very high
Human adenovirus 5	No	dsDNA	No	70-100	High
Bluetongue virus	No	dsRNA	No	60-80	Medium
Feline conjunctivitis virus	No	ssRNA	No	27-40	Medium-high
Simian adenovirus 15	No	dsDNA	No	70-100	High
PPV	No	ssDNA	No	18-24	Very high

* ss = single-strand; ds = double-strand.
† This general classification was provided by the CPMP guidelines.[20]
‡ NAT was implemented under an FDA approved investigational new drug (IND).

debris was removed by centrifugation, and the HIV infectious titer of the resulting cell-free supernatant was approximately 10^8 plaque-forming units per mL (PFU/mL). Titration of HIV was accomplished with a microplaque assay on MT-2 cells.[23]

Cell-free HIV-1 and HIV-2 clinical isolate

The low-passage, clinical viral stocks were harvested from culture supernatant from infected peripheral blood mononuclear cells (PBMNCs) and provided by Tektagen (Malvern, PA). The cell-free, clinical isolate HIV-1, strain Z84, was diluted 1:10 (vol/vol) into PLT concentrates to achieve the highest possible titer of approximately 10^3 to 10^4 tissue culture infectious dose ($TCID_{50}$) per mL. The cell-free, clinical isolate HIV-2, strain CBL20, was similarly diluted into PLT concentrates to achieve a highest possible titer of approximately 10^2 to 10^3 $TCID_{50}$ per mL.

PLT concentrate samples before treatment were titrated for viable virus on freshly prepared, phytohemagglutinin-stimulated PBMNCs in RPMI.[24] After treatment, 2 mL of PLT samples was assayed in 40 mL of PBMNCs. A total of two replicates, or 4 mL, were assayed. Inoculated PBMNCs were incubated at 37°C and split two to three times weekly for the duration of the study. On Day 11, the cultures were cocultivated with uninfected, phytohemagglutinin-stimulated PBMNCs at a cell ratio of 1:1. Supernatants were harvested on Days 7 and 16 for detection of viral replication with a ^{32}P-reverse transcriptase assay.[25] Titer was expressed as $TCID_{50}$ per mL.

Cell-associated HTLV-I and -II

HTLV-I, as infected cells 2060,[26] or HTLV-II, as infected C-19 cells (California Department of Health Services, Richmond, CA), were inoculated into PLT concentrates to a titer of approximately 10^5 focus-forming units (FFU) per mL. The infectivity of HTLV-I and -II was measured with the BHK21pA18G indicator cell line, which carries a β-galactosidase gene under the control of the long-terminal repeat region of the HTLV-I genome.[27] Trans-activating (*tax*) protein, produced as a result of infection with viable HTLV-I or HTLV-II, binds to the long-terminal repeat and initiates production of β-galactosidase, which can be detected by staining with an appropriate substrate. Foci of infected cells can be enumerated visually in a manner similar to plaques.

Heparin sulfate was added to all samples to a final concentration of 15 U per mL to prevent clotting during subsequent manipulations. Serial 10-fold dilutions were performed as necessary, and samples were inoculated into 24-well plates containing a monolayer of BHK21pA18G cells. The plates were incubated at 37°C in a humidified 5 percent CO_2 environment for 6 days to allow for the stimulation of β-galactosidase production induced by viral infection. The presence of HTLV-I or HTLV-II in each well was determined by staining with X-Gal (Sigma Chemical Co., St. Louis, MO) and enumerating HTLV-I- or HTLV-II-induced foci. Titer was expressed in FFU per mL.

DHBV

DHBV served as a model for HBV infection. DHBV was isolated from infected duck serum (gift from P. Marian). To measure viral titer, samples were serially diluted with PLT concentrate. Three- to 5-day-old Legarth Pekin hybrid ducklings (Metzer Farm, Gonzales, CA) were inoculated with samples by intravenous injection into the jugular vein. For each PLT preparation, six ducks were inoculated with 0.5 mL of the negative control and 12 ducks were inoculated with 6 mL of the undiluted PCT PLTs. All animals had been screened for congenital infection with DHBV. Nineteen days after inoculation, ducks were euthanized, and liver samples were collected for analysis. DNA was released from hepatocytes by mechanical homogenization in the presence of sodium dodecyl sulfate. Lysates were blotted onto a nitrocellulose membrane, and viral DNA was detected by hybridization with a radiolabeled DNA probe synthesized from cloned DHBV DNA on plasmid pD1.5G.[28] Autoradiograms were scored by comparing the hybridization signal of test samples with the intensity from samples containing known amounts of cloned viral DNA. For samples containing viable virus, DHBV titer was calculated with the method of Reed and Muench.[29] Titer was expressed as the infectious dose per mL.

HBV and HCV

Approximately $10^{3.5}$ to $10^{5.5}$ chimpanzee infectious doses (CID_{50}) of HBV (MS-2 strain, NIH repository, Bethesda, MD) or $10^{4.5}$ CID_{50} of HCV (Hutchinson strain, NIH repository) in PLT concentrate were evaluated.[30-32] Infectivity of these inocula was previously calibrated in chimpanzees naïve to HBV and HCV. The residual infectivity of PLT concentrates contaminated with HBV and HCV and subjected to PCT was measured in chimpanzees. The entire volume of each treated PLT concentrate (300 mL) was transfused into a nonimmune chimpanzee. The animal experiments were conducted in two phases with three chimpanzees. The dose of virus treated with PCT and order of transfusion were varied (Table 2).

In each phase of the evaluation, sequential serum samples were drawn from each animal over the course of 6 months for alanine aminotransferase (ALT) and apartase aminotransferase (AST) levels, seroconversion (hepatitis B surface antigen, anti-hepatitis B core antigen, anti-hepatitis B surface antigen, or anti-HCV), and the de novo appearance of the appropriate viral nucleic acids (HBV DNA or HCV RNA). Viral nucleic acids were detected by a PCR or reverse transcription-PCR assay. Liver biopsies

TABLE 2. The dose of human HBV (MS2 strain) and human HCV (Hutchinson strain) treated with PCT and order of transfusion in the two phases of the study*

Phase	Chimp X283	Chimp X286	Chimp X317
I	$10^{3.5}$ CID_{50}† HBV	$10^{4.5}$ CID_{50} HCV	$10^{5.5}$ CID_{50} HBV
II	$10^{4.5}$ CID_{50} HCV	$10^{4.5}$ CID_{50} HBV	$10^{4.5}$ CID_{50} HCV

* In each phase of the study, after transfusion with treated PLT concentrates, the animals were followed for a 6-month period for development of serologic, biochemical, molecular, and clinical signs of viral infection or disease.
† The infectivity of HBV and HCV is expressed in chimpanzee infectious dose (CID_{50}).

TABLE 3. Plaque assays used for detection of residual infectious virus*

Virus	Cell line used for the plaque assay	Medium†	Days to reading
BVDV	Bovine turbinate	EMEM-10% FBS	5
SARS-HCoV	Vero-E6	EMEM-10% FBS	2-3
Vaccinia virus	Vero-76	EMEM-10% FBS	3-5
Human adenovirus 5	A549	EMEM-10% FBS	7
Bluetongue virus	Bovine turbinate	EMEM-10% FBS	5
Feline conjunctivitis virus	Crandell feline kidney	EMEM-10% FBS	6
PPV	Porcine kidney-13	EMEM-10% FBS	10-12

* Samples containing viruses were serially diluted in cell culture medium as necessary, inoculated onto the appropriate cell layer, and incubated for approximately 1 hour at 37°C. The samples were aspirated and cell layers were overlayed with medium containing 0.75 percent agarose (Seaplaque, FMC Bioproducts, Rockland, ME). After the appropriate incubation at 37°C, plaque numbers were scored visually with or without the addition of an overlay containing neutral red (Sigma).
† EMEM = Eagle's minimal essential medium with Earle's salts; FBS = fetal bovine serum.

were obtained for histologic examination before and after each phase of the evaluation or following any ALT and AST elevation.

BVDV

BVDV (strain NADL, ATCC, Rockville, MD) was propagated in Madin-Darby bovine kidney (MDBK) cells (ATCC). Virus was harvested from culture supernatant and added to PLT concentrates to achieve a viral titer of approximately 10^5 PFU per mL. PLT samples were titrated for BVDV with a plaque assay on bovine turbinate cells (ATCC) (Table 3). Titer was expressed as PFU per mL.

Human CMV

Cell-associated CMV inocula were produced by propagating CMV, strain AD169 (ATCC), on human embryonic lung (MRC-5) cells (Cambrex, East Rutherford, NJ). Approximately 3.5×10^8 infected MRC-5 cells were added to CMV-seronegative PLT concentrates to yield an infectious viral titer of approximately 10^5 PFU per mL PLT concentrate. PLT samples were assayed to determine viable virus titer with a plaque assay on MRC-5 cells. For the treated samples, aliquots of 30 mL were assayed undiluted. Inoculated MRC-5 cells were incubated at 37°C for 7 days and then stained with neutral red. Titer was expressed as PFU per mL.

SARS-HCoV

The Urbani strain of SARS-HCoV (CDC, Atlanda, GA) was evaluated for sensitivity to PCT in PLT concentrates in two replicate experiments. The viral stock was prepared from culture supernatant of infected Vero-E6 cells (ATCC) after centrifugation to remove cell debris. The test unit contained 30 mL of PLT concentrate suspended in 35 percent plasma and 65 percent InterSol instead of a full-sized (300 mL) PLT unit. Initial and residual infectivity titers (PFU/mL) were determined by plaque assay on Vero-E6 cells (Table 3).

WNV

Virus was harvested from tissue culture supernatants of baby hamster kidney (BHK-21) cells (ATCC) transfected with a full genomic clone (Lineage I, pFL-WNV) of the parental WNV, strain 3356, isolated from the kidney of an American crow.[33] The tissue culture medium was clarified by centrifugation at $10{,}000 \times g$ and stored in aliquots at –80°C. The titer of the viral stock was approximately 1×10^9 to 5×10^9 PFU per mL.

The procedure for the WNV plaque assay was as previously described.[33] Samples were inoculated into Vero cells (ATCC). After incubation for 1 hour, the cells were overlayed with culture medium containing 0.6 percent Oxoid agarose (Basingstoke, Hampshire, UK). The plates were incubated for 2 days at 37°C and then overlayed with medium containing neutral red stain. The plates were incubated overnight to allow the plaques to develop and were visually read and counted on Day 3. Titer was expressed as PFU per mL.

Vaccinia virus

The IHD-W strain of Vaccinia virus (ATCC) was evaluated for sensitivity to PCT in PLT concentrates in three replicate experiments. The viral stock was prepared from culture supernatant of infected Vero-76 cells (ATCC) after centrifugation to remove cell debris. Test units contained 30 mL of PLT concentrate suspended in 35 percent plasma and 65 percent InterSol. Titers were determined by plaque assay on Vero-76 cells (Table 3). Titer was expressed as PFU per mL.

Human adenovirus-5

The human adenovirus 5 (gift from Onyx Pharmaceuticals Inc., Richmond, CA) was evaluated for sensitivity to PCT in a single experiment. The viral stock was prepared from culture supernatant of infected human embryonic kidney cells (HEK293) transformed with sheared human adenovirus 5 DNA (ATCC) after centrifugation to remove cell debris. The test unit contained a volume of 30 mL of 35 percent plasma and 65 percent InterSol, no PLTs. Titers (PFU/mL) were determined by plaque assay on lung carcinoma cells (A549, ATCC, Rockville, MD) (Table 3).

Bluetongue virus

The bluetongue virus stock (serotype 11, Station strain, ATCC, Rockville, MD) was prepared from the culture supernatant of infected baby hamster kidney-21 (BHK21) cells (ATCC) after centrifugation to remove cell debris. The titer of the stock was approximately 10^6 PFU per mL. The viral titer in PLT samples was titrated in bovine turbinate cells (ATCC) (Table 3). Titer was expressed as PFU per mL.

Feline conjunctivitis virus

The feline conjunctivitis virus (FC strain, ATCC) was prepared from the culture supernatant of infected Crandell feline kidney (CrFK) cells (ATCC) after centrifugation to remove cell debris. The titer of the stock was approximately $10^{8.7}$ PFU per mL. The viral titer in PLT samples was titrated in CrFK cells (Table 3). Titer was expressed in PFU per mL.

Simian adenovirus 15

The Simian adenovirus 15 (strain AP4398, ATCC) stock (SV-15) was prepared from the culture supernatant of infected fetal rhesus kidney (FRhK) cells (gift from the California Department of Health Services) after centrifugation to remove cell debris. The titer of the stock was approximately $10^{7.5}$ $TCID_{50}$ per mL. The viral titer in PLT samples was titrated in FRhK cells. Samples with additional serial dilutions were made in culture medium as necessary and inoculated onto 96-well plates containing FRhK cells. After 10 to 14 days of incubation at 37°C in a humidified 5 percent CO_2 incubator to allow for development of cytopathic effect, wells were evaluated for the presence or absence of cytopathic effect with the aid of a microscope, and sample titers were calculated in $TCID_{50}$ per mL.

PPV

The PPV stock (strain NADL-2, ATCC) was prepared from the culture supernatant of infected porcine kidney-13 (PK13) cells (ATCC) after centrifugation to remove cell debris. The titer of the stock was approximately $10^{7.5}$ PFU per mL. The viral titer in PLT samples was titrated in PK13 cells (Table 3). Titer was expressed in PFU per mL.

Parvovirus B19

Parvovirus B19–infected plasma samples (Alpha Therapeutics, Los Angeles, CA) were evaluated for sensitivity to PCT in 35 percent plasma and 65 percent InterSol. The test unit had a volume of 30 mL and no PLTs. Two replicate experiments were performed. Viable virus titer was determined by infection of CD34+ cells (All Cells, Berkeley, CA) and detection of progeny by ELISpot assay.[34] CD34+ cells obtained from granulocyte-macrophage–colony-stimulating factor–mobilized donor PBMNCs were treated with cytokines to induce proliferation and differentiation into CD36+ cells and then infected with serially diluted B19 and transferred to ELISpot plates that had been coated with a polyclonal antibody to B19 proteins (Dako, Carpinteria, CA). The ELISpot plates were incubated for 72 to 96 hours, and the cells were removed by washing. B19 antigens bound to the plates were detected by a monoclonal antibody to VP 1 and 2 (Chemicon, Temecula, CA). The spots were developed with a silver stain and counted visually with a stereomicroscope or on a plate reader (ImmunoSpot, Celluar Technologics, CTD, Cleveland, OH). The titer was expressed in spot-forming units per mL.

Statistical analysis

The level of viral inactivation was calculated as log-reduction with the formula

$$\text{Log-reduction} = \text{Log}(\text{pretreatment viral titer}/\text{posttreatment viral titer}).$$

After PCT, if no virus was detected, the viral titer was expressed as $<1/V$ infectious units, where V is the total PLT volume assayed. No virus detected indicated inactivation to below the limit of detection; thus the log-reduction was expressed as greater than the input titer. If no virus was detected in a sample volume significantly smaller than the volume of the PLT unit (300 mL), statistical analysis was performed with the Poisson distribution to calculate the concentration [C] of virus required to be detected with 95 percent probability according to the formula.

$$C = \ln(P_0)/-V,$$

where P_0, taken as 0.05 for 95 percent, represents the probability that no virus would be present in a sample of volume V.[20]

RESULTS

Enveloped viruses of varying size and nucleic acid content were uniformly sensitive to PCT with 150 µmol per L amo-

TABLE 4. Inactivation measured by infectivity assays for enveloped viruses after treatment with 150 µmol per L amotosalen and 3 J per cm² UVA

Virus	Pretreatment titer	Infectivity units	Volume (mL) assayed at 3 J/cm²	Log-reduction*	Log-reduction (95% CI)
HIV-1					
Cell-free	$10^{6.2 \pm 0.1}$	PFU/mL	3	>6.7 ± 0.1	>6.2 ± 0.1
Cell-associated	$10^{6.1 \pm 0.3}$	PFU/mL	3	>6.6 ± 0.3	>6.1 ± 0.3
Clinical HIV-1, Z84	$10^{3.3 \pm 0.4}$†	$TCID_{50}$/mL	4	>3.9 ± 0.4	>3.4 ± 0.4
Clinical HIV-2, CBL20	$10^{2.3 \pm 0.3}$†	$TCID_{50}$/mL	4	>3.0 ± 0.3	>2.5 ± 0.3
DHBV (a model for HBV)	$10^{6.0 \pm 0.6}$	ID_{50}/mL	6	>6.7 ± 0.6	>6.2 ± 0.6
HBV (MS2)	$10^{3.5\text{-}5.5}$	CID_{50}	300	>5.5	>5.5
BVDV (a model for HCV)	$10^{4.8 \pm 0.7}$	PFU/mL	49	>6.5 ± 0.7	>6.0 ± 0.7
HCV (Hutchinson)	$10^{4.5 \pm 0.0}$	CID_{50}	300	>4.5	>4.5
HTLV-I	$10^{4.7 \pm 0.1}$	FFU/mL	9	4.7 ± 0.1‡	4.2 ± 0.1‡
HTLV-II	$10^{5.1 \pm 0.1}$	FFU/mL	9	5.1 ± 0.1‡	4.6 ± 0.1‡
CMV	$10^{4.9 \pm 0.3}$	PFU/mL	30	>6.4 ± 0.3	>5.9 ± 0.3
WNV	$10^{5.7 \pm 0.4}$	PFU/mL	2	>6.0 ± 0.4	>5.5 ± 0.4
SARS-HCoV§	$10^{5.5 \pm 0.1}$	PFU/mL	3, 13	>6.3	>5.8
Vaccinia virus§	$10^{5.4 \pm 0.2}$	PFU/mL	0.6	>5.2 ± 0.2	>4.7 ± 0.2

* > = inactivation to below the limit of detection. No virus was detected in the volume assayed.
† Highest possible titer.
‡ No " >" was assigned because there was a low level of naturally occurring β-galactosidase expression in the BHK21 indicator cells, and it was not possible to differentiate whether the few β-galactosidase-expressing cells present in wells inoculate with treated samples were a result of this natural β-galactosidase expression or whether they were truly infected with HTLV-I or -II. The conservative approach assumed that all β-galactosidase production was the result of HTLV infection.
§ Inactivation experiments were performed with 30-mL aliquots of PLT concentrates instead of the full-sized (300 mL) units. In addition, although four replicate experiments were performed for other viruses, only two and three replicates were performed for SARS-HCoV and vaccinia virus, respectively.

tosalen and 3 J per cm² UVA in PLT concentrates (Table 4). High levels of a broad spectrum of enveloped viruses were inactivated to the limit of detection. When feasible, inactivation studies were designed to achieve a 6-log dynamic range in infectivity. For clinical HIV isolates, viral stocks with the highest available titer were used and the level of inactivation was at the limit of the dynamic range achievable for these clinical isolates. Inactivation of HIV clinical isolates was shown to be as sensitive to PCT as laboratory adapted strains.

The inactivation level, expressed as log-reduction, was first calculated with the experimental findings regardless of the sample volume (Table 4, Column 5). When the sample volume used for the measurement of residual viral titer was significantly lower than the treatment volume of 300 mL per unit PLT concentrate, the Poisson distribution was used to estimate the minimum concentration of virus that would have to be present in the PLT unit to be detected with 95 percent probability in a smaller sample volume. This led to a conservative and lower limit on the viral log-reduction (Table 4, Column 6).

When inactivation below the detection limit was obtained, log-reduction was reported as greater than the input titer. For HTLV assays, there was a low level of naturally occurring β-galactosidase expression in the BHK21 indicator cells, and it was not possible in this study to differentiate whether the few β-galactosidase-expressing cells present in wells inoculated with treated samples were a result of this natural β-galactosidase expression or whether they were truly infected with HTLV. The conservative approach was used in the calculation of inactivation by assuming that all β-galactosidase production was the result of infection.

For HBV and HCV, inactivation was demonstrated with the well-documented and characterized MS2 and Hutchinson strain, respectively. The highest available titers of calibrated viral stocks were used for these studies. Infectivity was measured in nonimmune chimpanzees. In both phases of the study, none of the animals showed serologic, viral nucleic acid, biochemical, or histologic evidence of viral hepatitis during a 6-month follow-up. The results demonstrated inactivation of up to greater than 5.5 logs of HBV and up to greater than 4.5 logs of HCV. To further evaluate the efficacy of PCT for hepatitis viruses and to expand the dynamic range for HBV and HCV, model viruses were used. DHBV has been chosen as a model for HBV because of its extensive genetic and ultrastructural homology with HBV.[35] BVDV was selected as a model for HCV because of ultrastructural similarity between HCV and BVDV.[36] By use of these model viruses, greater than 6 logs of inactivation were demonstrated.

CMV was shown to be sensitive to PCT. Previous studies have demonstrated that high levels of CMV inactivation could be achieved with an efficacy margin of more than 100-fold.[17]

TABLE 5. Inactivation measured by infectivity assays for nonenveloped viruses after treatment with 150 µmol per L amotosalen and 3 J per cm^2 UVA

Virus*	Pretreatment titer	Infectivity units	Volume (mL) assayed at 3 J/cm^2	Log-reduction†	Log-reduction (95% CI)
Human adenovirus 5‡	$10^{7.1}$	PFU/mL	0.04	>5.7	>5.2
Parvovirus B19‡	$10^{4.5\text{-}5.5}$	SFU§/mL	0.4	4 to > 5.5	3.5 to > 5.0
Bluetongue virus	$10^{4.8 \pm 0.2}$	PFU/mL	30	6.1 to 6.4‖	5.6 to 5.9‖
Feline conjunctivitis virus	$10^{7.8 \pm 0.1}$	PFU/mL	<1	1.7 to 2.4¶	1.7 to 2.4¶
Simian adenovirus 15	$10^{3.4 \pm 0.1}$	$TCID_{50}$/mL	<1	0.7 to 2.3¶	0.7 to 2.3¶
PPV	$10^{4.7 \pm 0.1}$	PFU/mL	<1	0	0

* A single preliminary experiment with hepatitis A virus was carried out with 4′-aminomethyl-4,5′,8-trimethylpsoralen, a similar psoralen to amotosalen, under different treatment conditions demonstrated resistance of hepatitis A virus to psoralen PCT. The data are not included in this table.

† > = inactivation to below the limit of detection. No virus was detected in the volume assayed.

‡ Inactivation experiments were performed with 30-mL aliquots of 35 percent plasma and 65 percent InterSol instead of the full-sized (300 mL) PLT units. There was a 15- to 30-minute incubation period between addition of 150 µmol per L amotosalen and UVA illumination. In addition, although four replicate experiments were performed for other viruses, only one and two replicates were performed with human adenovirus 5 and parvovirus B19, respectively.

§ SFU = spot-forming units.

‖ The inactivation results for the four replicates were ≥6.1, 6.4, 6.4, and ≥6.4 (at a conservative range of 6.1-6.4), corresponding to 0, 1, 1, and 0 residual plaques, respectively. Because of the low level of detection, the Poisson distribution was used to estimate the minimum concentration of virus that would have to be present in the PLT unit to be detected with 95 percent probability in a 30-mL volume. This led to the lower limit on the log-reduction (range 5.6-5.9).

¶ The residual number of viable virus was significant (>95% confidence).

Nonenveloped viruses demonstrated variable inactivation with PCT with 150 µmol per L amotosalen and 3 J per cm^2 UVA in PLT concentrates (Table 5). Although the most relevant, blood-borne, human nonenveloped viruses, such as parvovirus B19 and human adenovirus 5, were sensitive to PCT, inactivation of other nonenveloped viruses did not reach the limit of detection. Of all nonenveloped viruses evaluated to date, only hepatitis A virus and PPV have shown resistance to PCT.

DISCUSSION

An effective pathogen inactivation system for blood components must have a broad-spectrum inactivation capability while maintaining the biologic properties of the blood components. In the development of the PCT system for PLTs described in this report, studies were performed to demonstrate inactivation of bacteria, viruses, protozoa, and white blood cells (WBCs) in PLT concentrates.[15,16,19,37,38]

To demonstrate the efficacy of virus inactivation, a three-pronged approach was used. The first was to demonstrate inactivation of viruses that are most relevant to blood transfusion and are routinely tested for in blood donations, including HIV-1, HIV-2, HBV, HCV, HTLV-I, and HTLV-II. Although testing for CMV is not routinely required, it is included because of its significance for immunocompromised patients. The second was to evaluate the inactivation of viruses that are of emerging interest, including parvovirus B19, WNV, SARS-HCoV, and vaccinia virus. The third was to use model viruses that represent a broad spectrum with respect to envelopes, genome and capsid types, virion sizes, and resistance to treatment, including DHBV, BVDV, bluetongue virus, feline conjunctivitis virus, simian adenovirus 15, and PPV. Use of model viruses increased the dynamic range of an infectivity assay where use of authentic human viruses may not be practical or when infectivity assays were lacking.

The FDA has suggested that ideally a pathogen reduction (inactivation) treatment system would have the ability to reduce the pathogen load in a blood product by 6 to 10 logs, because some viral titers in the window period of infected donors can reach levels of 10^8 to 10^{10} genome equivalents (geq) per mL.[21] It should be noted that a viral titer in geq per mL is measured by quantitative PCR of a small fragment (100-200 bases) of the viral genome. Whether 1 geq corresponds to 1 infectious unit of a virus has not been unequivocally demonstrated. Published ratios of geq to infectivity were between 10^4:1 and 10^5:1 for parvovirus B19 and 360:1 for SARS-HCoV.[39-41] Therefore, experiments conducted in this report were targeted to have a 6-log dynamic range in infectivity of a virus whenever possible. In contrast to geq titers, infectivity titers are measures of intact, functional viral genomes, which are required for replication and disease transmission. Reduction in viral infectivity titers is directly related to the risk reduction of viral transmission.

The aggregate results summarized in this report demonstrate that all enveloped viruses routinely screened in blood banks were sensitive to PCT, and high levels of inactivation were obtained. PCT, however, did not inactivate

all nonenveloped viruses evaluated. Bluetongue virus was more sensitive to inactivation than feline conjunctivitis virus or simian adenovirus 15. Nonenveloped viruses with tight capsids, for example, PPV, are resistant to inactivation. Because PCT is a nucleic acid targeting method, PCT will not impact the transfusion risk due to prions, which contain no nucleic acid and cause variant Creutzfeldt-Jakob disease and possibly chronic wasting disease.[42,43]

Although PCT did not reduce the titers of PPV, the human erythrovirus B19 was inactivated. Other investigators also reported disparate results for these two viruses, suggesting that PPV is not an appropriate model virus for B19.[39,44] By use of an ELISpot assay[34] that detects and quantifies infectious parvovirus B19 particles, PCT inactivation of up to greater than 5 logs of infectious B19 has been demonstrated. The effect of PCT on parvovirus B19 can be improved when amotosalen is incubated with infected PLTs for 1 hour or more, in which case up to 5.8 logs can be inactivated in PLT concentrates.[45] The incubation period allowed penetration of amotosalen through the viral capsid and subsequent reaction with the viral genome upon UVA illumination.

Emerging viruses are a persistent threat to blood transfusion safety. In recent years, the most important examples of emerging viruses have been WNV, introduced into North America, and SARS virus, reported in Asia.[46] Vaccinia virus, a model for smallpox virus, is of interest because the blood supply could represent a soft target for bioterrorists. New viruses continue to emerge at a rate of one every 2 to 3 years with a potentially damaging virus transmitted through blood every 5 years.[47] Testing remains a reactive approach to blood safety. The new viruses must be identified before sensitive tests can be developed. Although the time to develop an assay has shortened as the technology improves, the time it takes for a new virus in the blood supply to be recognized as a threat may be significant as demonstrated by the current WNV outbreak in the United States.[48] Furthermore, all tests have a sensitivity threshold that can allow contaminated units to escape detection. For WNV, in spite of the institution of NAT in 2003 under an FDA-approved investigational new drug application, six transfusion-transmitted WNV infections were confirmed.[49] In addition, testing only applies to a small percentage of the organisms that may infect blood; a number of bacteria and protozoan parasites, including the agents of Lyme disease, babesiosis, malaria, Chagas disease, and leishmaniasis, are known to be transmitted by blood transfusion, but no routine testing has been implemented in Europe or the United States.

In contrast, pathogen inactivation with PCT is a proactive approach to blood safety. Inactivation of up to greater than 6 logs of viral infectivity has been demonstrated for a broad spectrum of viruses. A previous publication has reported inactivation of up to greater than 6 logs of a wide variety of bacteria.[19] In addition, WBCs contain nuclear DNA and are targets for inactivation. Previous studies showed that PCT induced one amotosalen-DNA adduct in approximately every 83 bp.[37] Therefore, PCT can potentially offer a one-step process that inactivates contaminating pathogens as well as WBCs. These advances in pathogen inactivation may ultimately influence the strategy of testing versus inactivation. In a recent article, 15 international experts expressed their opinions on whether any of the current tests on donor blood can be omitted when PCT is used.[50] Most do not think pathogen inactivation and testing are mutually exclusive. No current tests can be omitted until pathogen inactivation methods for all blood components are available. Suggested possible exceptions may include omission of tests for syphilis, CMV, bacterial testing for apheresis PLT concentrates, and gamma irradiation. The development of new tests for emerging pathogens may be eliminated or delayed. However, owing to the concerns of the geq titer of greater than 10^{10} for viruses such as B19 in the window period, the conservative approach would be to retain the most sensitive current tests to assure that levels of viremia are well below the documented pathogen inactivation effectiveness threshold. Some new tests may be required, even in the setting of universal pathogen inactivation, to safeguard against various high-titer pathogens that could potentially "breakthrough" pathogen inactivation.

Among the currently licensed pathogen inactivation and/or reduction methods for plasma and plasma derivatives, such as solvent/detergent (S/D),[51] methylene blue,[52] heat inactivation,[53] and ultrafiltration,[54] the antiviral effect varies among each approach. S/D is not effective against nonenveloped viruses, and methylene blue is not effective in inactivating nucleated cells and, correspondingly, intracellular and cell-associated virus.[55] Heat inactivation is effective against some enveloped viruses, but parvovirus B19 transmission has been reported for heat-treated clotting factor concentrates.[56] Ultrafiltration may offer the possibilities of reducing the viral load of small nonenveloped viruses that are difficult to inactivate by chemical methods. These licensed pathogen reduction methods, however, are only for cell-free blood products; none is applicable to blood cellular component PLT concentrates. The PCT system described in this report represents the first pathogen inactivation method for PLT concentrates with a comprehensive broad-spectrum activity. The PCT system has been CE Mark approved and is in clinical use in several countries in Europe. Another system involving riboflavin photochemistry in development for PLT concentrates has not demonstrated this degree of activity.[57]

In conclusion, results in this report show that PCT is effective in inactivating viruses from 10 different families, including enveloped, nonenveloped, DNA- or RNA-containing, proviral, cell-free, and cell-associated viruses and, therefore, offers the potential to prospectively pre-

vent the majority of PLT transfusion–associated viral diseases.

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