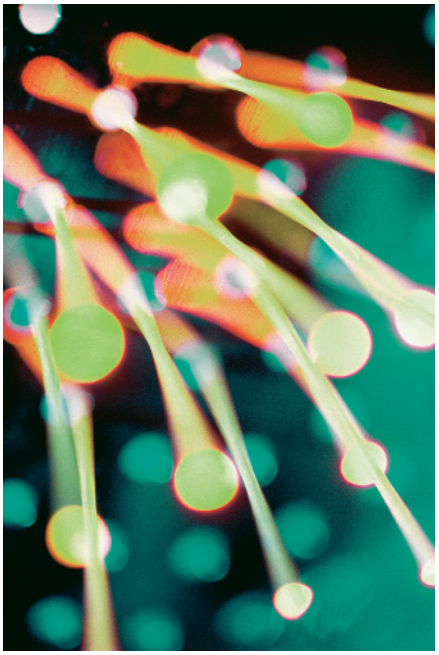

Inactivating Adventitious Viruses While Preserving Biological Activity

Treating Fetal Bovine Serum with Pulsed Ultraviolet Light

Masoud Hosseini, Mary Sawyer, Carrie Schore, Joseph Vu, Krystyna Trzepla-Nabaglo, Cecilia Pina, Wayne Smith, Manuel Lagunas-Solar, and Bennie I. Osburn

New evidence suggests the West Nile virus can be transmitted during blood transfusion from an asymptomatic donor. However, it is now possible to use pulsed ultraviolet laser light to inactivate viruses such as West Nile at large production scales. These experimental data show that using laser light on virus-treated media can render biological products free of contaminating viruses without compromising the biological activity essential to cell cultures.

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In the early 1900s, ultraviolet radiation (UV) was found to be effective in controlling living organisms (1). However, light from this portion of the electromagnetic spectrum damages cellular DNA, compromising the biological activity essential to cell cultures. Recently the availability of a variety of new, pulsed, high-peak power light sources with narrow-band UV and broad-band UV visible (vis) infrared (IR) spectra, such as Xenon (Xe) flash lamps, has prompted investigations aimed at developing new uses for pulsed power in biomedicine and industry. Excimer UV sources, such as lasers and lamps (2–5), provide state-of-the-art performance and high reliability, allowing monochromatic UV radiation to be used in a variety of applications (6–8).

Our first experiment demonstrates the ability of laser technology to inactivate viruses by delivering nonadditive, ultrashort pulses of high-megawatt laser light (248 nm) at high repetition rates. The second study shows that fetal bovine serum (FBS) remains unaffected in its ability to support the growth of different cell lines even when treated with pulsed ultraviolet (PUV) exposures greater than necessary for viral inactivation. (The names for all viruses abbreviated in this article are listed in the "Viral Contaminants" sidebar.)

Methods of Viral Inactivation

Viral contamination of biological products is of concern to public health officials, biologic industries, and research and diagnostic laboratories. Viral contaminants that have been documented in blood or blood-derived products of animal origin include BTV, BVDV, PI-3, IBRV, BEV, PPV, and ALV (9–14). Contamination can also come from viruses of human origin, including HAV, HBV, HCV, HEV, HGV, PV-B19, HIV, SV-40, HTLV, and CMV (15,16).

A variety of methods have been used to remove or inactivate contaminating viruses from blood or blood components (17–20). Some of these methods are effective, but all have significant disadvantages.

Viral Contaminants

ALV	avian leukosis virus
BEV	bovine enterovirus
BTV	blue tongue virus
BVDV	bovine viral diarrhea virus
CMV	cytomegalovirus
HAV	hepatitis A virus
HBV	hepatitis B virus
HCV	hepatitis C virus
HDV	hepatitis D virus
HEV	hepatitis E virus
HGV	hepatitis G virus
HIV	human immunodeficiency virus
HTLV	human T lymphocyte virus
PV-B19	human parvovirus B19
IBRV	infectious bovine rhinotracheitis virus
PI-3	parainfluenza type 3
PPV	porcine parvovirus
SV-40	simian virus-40
WNV	West Nile virus

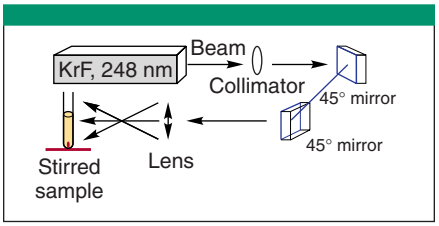

Figure 1. Experimental setup for pulsed ultraviolet treatment

Physical methods such as filtration technology have merit. But small virus particles require such small filter sizes that high molecular weight proteins are also filtered out. A centrifuge cannot always be used to clearly distinguish between viruses and proteins because of the different sedimentation patterns. And centrifuges are impractical for large-scale processing of blood products.

Precipitants such as ethanol remove viruses, but the inactivation must be repeated and requires the use of additional methods (21).

Heating is effective if the temperature is sufficient to fully inactivate the agents, however some viruses, such as parvovirus B19, are resistant to heat inactivation (22).

Radiation, including the use of gamma irradiation or UV light, has not experienced much success. Gamma radiation sufficient to inactivate viruses has adverse effects on proteins such as clotting factors (23).

Chemical sterilization using products such as 1% tri-(*n*-butyl) phosphate and 1% Triton X-100 or β-propiolactone (β-PL) is reasonably effective except for the inability to inactivate nonlipid-enveloped viruses (15). This treatment does not appear to have adverse effects on clotting proteins, but chemical sterilization can take as long as 30 hours and must be followed by chromatography to completely remove all chemical traces.

Biological methods using neutralizing antibodies (NAbs) can be used only with known viruses that produce NAbs (24). NAbs effectively inactivate their homologous virus; but dissociation of the NAb–virus complex can be a concern, as can the preparation and sterility of such NAbs.

Immunoaffinity chromatography is a combination of chemical and physical methods for removing viruses from blood-derived products. It requires specific immunoglobulins and is limited to inactivating one specific virus per immunosorbent (20).

Photochemical sterilization combines UV irradiation and chemical agents such as psoralens or β-PL. Many viruses can be photochemically inactivated by using psoralens because, in the presence of long-wave UV-light, psoralens photoreact with the nucleic acid of viruses. Picornaviruses are one group of viruses that remain unaffected (25).

Virus Sources, Methods, and Titers

Our preparation of the stock viruses used in these experiments varied with the propagation requirements specific to each virus. The cell lines, handling, and titer determinations that we used are described here; full virus names are detailed in the "Viral Contaminants" sidebar.

Stock Preparation

BTV. We used baby hamster kidney (BHK-21) cells, purchased from American Type Culture Collection (ATCC, www.atcc.org). These cells were infected with a BTV-10 isotope plaque, picked in this laboratory and harvested when the cytopathic effect was at 75%. The virus–cell material was sonicated (Misonix, www.misonix.com) and centrifuged to remove cellular debris. We stored the supernatant containing BTV at 4 °C. The titer of this stock virus was $10^{6.3}$ tissue culture infective dose ($TCID_{50}$) per mL.

IBRV. Seed IBRV from ATCC was propagated using Madin–Darby bovine kidney (MDBK) cells (ATCC). We harvested when the cytopathic effect (CPE) was greater than 75%. The flask was then frozen (−70 °C) and thawed three times and then centrifuged to separate cell debris from the supernatant containing IBRV. The virus was aliquotted and stored at −70 °C. The titer of the stock virus was $10^{7.46}$ $TCID_{50}$/mL.

BVDV. Cytopathic stock BVDV (cell line Kentucky 22) from Biological Research Faculty and Facility (BRVV, Ijamsville, MD) was stored at −70 °C. The titer of this stock virus was $10^{7.12}$ $TCID_{50}$/mL.

PPV. Each virus stock was diluted in fetal bovine serum (FBS) to one part virus for five parts medium. The FBS used throughout these experiments was from a lot proven to be free of contaminating viruses, including noncytopathic BVDV (Lot No. 2567 from HyClone, www.hyclone.com). We reconfirmed that this virus was absent in our laboratory. The stock virus of BTV was similarly diluted in phosphate-buffered saline (PBS) for this study.

We prepared three replicates of 4-mL samples in quartz tubes for each stock virus. The replicate samples were treated with pulsed ultraviolet (PUV), and the contents pooled and stored appropriately. Two independent analyses for the titer determinations were performed in duplicate on each of the pooled samples. The results of each duplicate analysis were averaged, and the average of each group represents a single data entry.

Titration

Virus activity was determined by titration (tenfold dilutions) in 96-well microplates using cell lines for each virus: We used Vero cells for BTV, MDBK cells for IBRV, bovine turbinate (BT) cells for BVDV, and pig kidney (PK-15) cells for PPV. The virus titers were calculated using the Spearman–Karber method (1) and expressed as a 50% $TCID_{50}$ per mL. Log_{10} reduction of the titer was calculated for each energy dose by subtracting the posttreatment titer from the titer of the pretreatment sample. Pretreatment titers were different for each virus. Therefore, a log_{10} reduction percent was used as a basis of comparison for the effects of treatments. Linear regression graphs were constructed (using Sigmaplot from SPSS, Inc., www.spss.com), and the coefficient of determination (r^2) values determined.

The results of the virus inactivation experiment are described in the "Viral Inactivation Results" sidebar.

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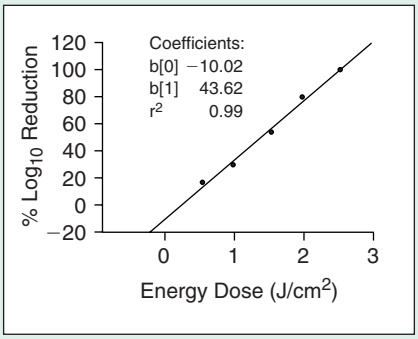
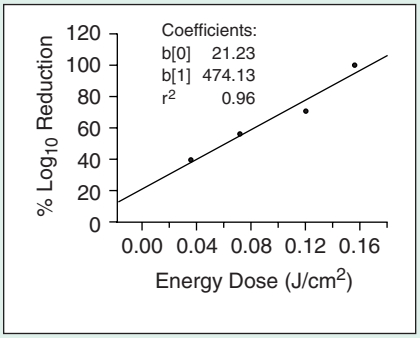
Viral Inactivation Results

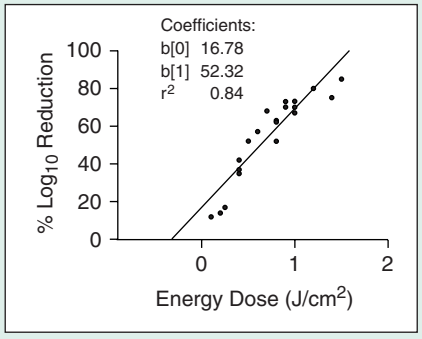
The figures below show that relatively similar energy doses are required to reduce the log_{10} virus titer of three of the viruses studied. The titers of IBRV, BVDV, and PPV were each reduced at relatively low doses of energy. BTV results, however, suggest that more energy is required for inactivation in FBS than in PBS. The graphs below are linear regression graphs plotting percent log reduction against energy dose in joules per cm^2.



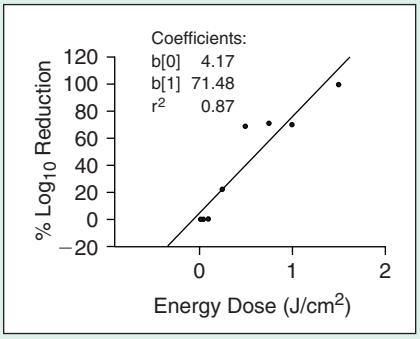
BTV in FBS. The titer of BTV suspended in FBS was $10^{4.99}$ $TCID_{50}$/mL. Extrapolating from the plot of result data points, an 80% log_{10} virus titer reduction is estimated at 2.0 J/cm^2 of laser energy. At 2.55 J/cm^2 of laser energy, 100% log_{10} reduction results.



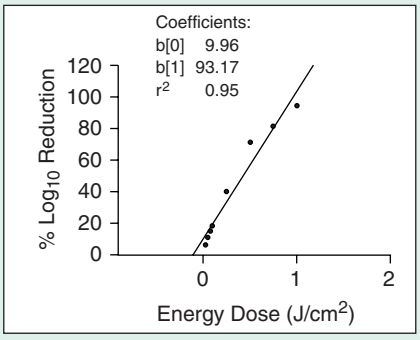
BTV in PBS The titer was $10^{4.55}$ $TCID_{50}$/mL. An 80% log_{10} virus titer reduction was estimated at 0.13 J/cm^2, and 0.16 J/cm^2 reduced 100%. The energy required to inactivate BTV was 15–16 times less at the 80% and 100% log^{10} reduction levels than when BTV was suspended in FBS.



IBRV in FBS. The titer was $10^{6.58}$ $TCID_{50}$/mL. An 80% log_{10} virus titer reduction is estimated at 1.2 J/cm^2 of laser energy. The maximum energy dose attempted in this series was 1.5 J/cm^2 . Extrapolating from the plot indicates that 100% log_{10} virus titer reduction would occur at doses of 1.6 J/cm^2.



BVDV in FBS. The titer of the pretreated BVDV suspended in FBS was $10^{5.18}$ $TCID_{50}$/mL. An 80% log_{10} virus titer reduction is estimated at 1.1 J/cm^2 of laser energy. Virus titer reduction of 100% was achieved at doses of 1.5 J/cm^2. Extrapolation from the plot indicates that inactivation may have occurred below that energy dose.



PPV. The titer of the pretreated PPV suspended in FBS was $10^{7.30}$ $TCID_{50}$/mL. An 80% log_{10} virus titer reduction was achieved by exposure to 0.75 J/cm^2 of PUV; A 94% log_{10} virus titer reduction is achieved at 1.0 J/cm^2.

Lasers and Viruses

Our goal was twofold: to test the ability of PUV laser light to inactivate a panel of viruses and then to determine whether the growth rate and activity of several cell lines remained unaffected when FBS media was exposed to UV radiation. The equipment and viruses we used are listed here; the materials and methods are detailed in the "Virus Sources, Methods, and Titers" sidebar.

Laser parameters. For our experiments, we used a Compex 110 Excimer laser (Lambda Physik AG, www.lambdaphysik.com) operating in krypton fluoride (KrF) mode at five (248 nm) electron volts (eVs) per photon. Pulse duration was 30 nanoseconds (ns). To ensure that the entire sample was exposed to the laser light, we had to determine the desired beam area and geometry. We adjusted the shape of the beam by passing it through a variable circular collimator, redirected it by using 45° UV-reflecting mirrors, and then expanded it by passing it through the appropriate diverging lenses (see Figure 1).

The laser beam energy was measured as the laser exits and varied from 150 to 300 mJ/pulse. To deliver the desired energy exposure to the samples, we operated the laser system at a variable pulse repetition rate ranging from one to 10 Hz. As a control, we placed 4-mL samples in quartz tubes of known reflectance and absorption characteristics at a fixed distance from the light source. During treatment, the samples were stirred with a magnetic stir-bar to

Does It Still Grow Well?

Our second goal was to determine whether FBS media exposed to PUV laser light still produced viable cell cultures. For this test, we prepared FBS (Lot No. 2567, HyClone) for laser treatment, using a centrifuge for 20 minutes at 1,000*g* to remove protein debris that comes from clotting factors that remain in FBS. The FBS was then exposed to 10 J/cm^2 pulsed UV light at 248 nm.

Cell lines used. For evaluation of laser-treated FBS, we used three different mammalian cell lines to evaluate the ability of laser-treated FBS to support growth in Vero, BHK-21, and myeloma (P3X63Ag8.653 murine myeloma) (1) mammalian cell lines (from ATCC, www.atcc.org). We also used an insect cell line, *Spodoptera frugiperda* (Sf-9) from Invitrogen Company (www.invitrogen.com).

Initially, each cell line was divided into two identical samples. One preparation was for continuous passage in medium supplemented with untreated FBS, the other with laser-treated FBS. Each of the two preparations was passed in triplicate for at least 14 successive passages without pooling. At each passage, viable cells and total number of cells were determined for each preparation using trypan-blue exclusion and recorded individually for each triplicate.

Each cell line passed at a defined time with a specific seeding concentration. BHK-21 and Vero cell lines were dissociated by trypsin; myeloma and Sf-9 cells were physically dislodged.

Vero and BHK-21 cell lines were treated with minimum essential media (MEM) with 1% L-glutamine and 1% penicillin/streptomycin (P/S) 10,000 units/10,000 mg/mL added. The myeloma cell line was treated with MEM and RPMI 1640 reagent prepared with 1% L-glutamine, 1% P/S, 1% sodium pyruvate, and 1% nonessential amino acids. The Sf-9 cell line was prepared with Grace's insect medium, with L-glutamine and 0.2% Gentamycin added. The medium for all cell lines was supplemented with either 10% untreated (for control samples) or 10% PUV-treated FBS. We compared the ability of the progeny of Vero cell lines to produce virus after 14 successive passages on medium using the untreated and laser-treated FBS. We pooled three replicates of Vero cells separately and infected those with cell culture-adapted BTV-10. The virus titer was determined on each replicate by titration in the microplate system.

Results

Based on viable cell counts, the average cell propagation for BHK-21 and Vero cells after 14 passages, for myeloma cells after 16 passages, and for Sf-9 cells after 18 passages was 98%, 99%, 91%, and 94% respectively compared with the related control cell lines. Based on total cell count, the average cell propagation for BHK-21, Vero, myeloma, and Sf-9 cell lines after similar passage levels was 98%, 98%, 99%, and 98% respectively of control cell lines. The average titer of the BTV-10 produced by the Vero cell progeny grown on medium with laser-treated FBS was 0.8 $\log_{10}$ higher than the titer of the BTV-10 produced by the Vero cell progeny grown on medium with untreated FBS.

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prevent the samples from absorbing varying irradiation.

Viruses and cell lines used. We selected the virus panel for this study based on the virus's structure and on the likelihood of its contaminating biologic products. Our preparation of the stock viruses used in these experiments varied with the propagation requirements specific to each virus. The cell lines, handling, and titer determinations that we used with each virus in the panel are detailed in the "Virus Sources" sidebar; full virus names are detailed in the "Viral Contaminants" sidebar. Each virus in our panel is described here briefly.

- *BTV*, the prototype of the genus *Orbivirus* within the family of Reoviridae, is an insect-transmitted disease agent of ruminants and dogs (9). BTV is a double-stranded RNA (dsRNA) virus with icosahedral symmetry and a diameter of 65–80 nm (26,27). It has the ability to pass through the bovine placenta, infecting the fetus and contaminating FBS (9).
- *IBRV*, a member of the Herpesviridae family, is one of the major pathogens of cattle. IBRV has a double-stranded DNA (dsDNA) genome. It is an enveloped, icosahedral nucleocapsid virion, 150 nm in diameter (28). This virus has also been detected as a contaminant of FBS (11).
- *BVDV* is a member of genus *Pestivirus* within the family of Flaviviridae. The genome consists of a ssRNA molecule. The icosahedral core is surrounded by a lipid envelope, and the virus is 40–60 nm in diameter. There are two biotypes, cytopathic and noncytopathic. BVDV can infect the bovine fetus through the placenta during the viremic stage (29). It is well documented that noncytopathic BVDV is the major viral contaminant of FBS. Investigators involved with cell culture or vaccine production are keenly aware of the potential problems emanating from the presence of this adventitious virus in products used in related industries (10). BVDV is closely related to human HCV. As a member of the Flaviviridae family, BVDV is similar to West Nile virus (WNV) of the genus *Flavivirus*. Recent evidence suggests that WNV infection can be transmitted through blood transfusion from an asymptomatic donor.
- *PPV* is a member of the Parvoviridae family. PPV is 20 nm, with a single stranded DNA (ssDNA) genome containing only three proteins (30). PPV has been documented crossing the porcine placenta and causing contamination of porcine fetal kidney cell cultures (11). Human parvovirus B19 has been identified as a contaminant of importance in transfusions of plasma-derived products (22).

The results of the virus inactivation experiment are described and summarized in the "Viral Inactivation Results" sidebar.

Viral Inactivation Experiment

In our experiments to test the ability of PUV laser light to inactivate a panel of viruses, there appeared to be no relationship between the susceptibility of a virus to UV energy and its nucleic acid structure, virion, or genome size. The substrate in which the virus was suspended, however, did have a consistent effect on the amount of energy required to inactivate the virus. BTV suspended in phosphate-buffered saline (PBS) required 15–16 times less energy to reduce the $\log_{10}$ titer than when suspended in FBS. The most obvious explanation is that the more proteinaceous FBS substrate absorbs more of the energy delivered than the aqueous PBS solution, so it takes more energy to inactivate a virus in the FBS substrate than in PBS.

To confirm that finding, we used PUV light at 308 nm (xenon chloride) to inactivate BTV suspended in PBS and in FBS. In PBS, our results showed viral reduction of 3.1 $\log_{10}$ $TCID_{50}$ requiring 5.0 J/cm^2. In FBS, only 1.1 $\log_{10}$ reduction was achieved at 10.0 J/cm^2 (unpublished data). This implies that UV light at 308 nm is much less effective than at 248 nm for inactivating BTV. That finding is supported in a report (31) that found UVC (wavelengths lower than 280 nm) to be more effective than UVB (280–320 nm) at inactivating nonenveloped viruses.

Does It Grow Well?

Our second goal was to determine whether FBS media exposed to PUV laser light still produced viable cell cultures. The cell lines and their sources, the methods, and the cell culture results are described in the "Does It Still Grow Well" sidebar. Our results showed that even with PUV exposures greater than that necessary for inactivating the viruses, the ability of the treated FBS to support growth of different cell lines was unaffected.

Avian cell lines were not included in our study because most continuous avian cell lines require either a combination of FBS and chicken serum or are able to sustain minimum growth only with high concentrations of FBS alone (some at 15%). Our experimental parameters were limited to cell lines requiring 10% FBS for sustained growth for at least 14 successive passages of PUV laser light.

Overall, based on viable cell count, all cell lines grown on laser-treated FBS exhibited minimal inhibitory effects when compared with nonlaser FBS-treated control cell lines. Total cell counts revealed no differences in the number of cells in each paired cell line. Other studies (32) reported that FBS treated with UV light at 248 nm was able to support cell growth.

Biochemical or electrophoretic analyses of the laser-treated FBS components would be complementary tests that would provide information on more subtle effects of laser energy on FBS. In unpublished results (Baxter BioScience Laboratories, www.baxter.com), high performance liquid chromatography (HPLC) and polyacrylamide gel electrophoresis (PAGE) were used to analyze PUV-treated human serum albumin (HAS). At an energy level of one J/cm^2, HPLC and PAGE assays revealed that 99% of the untreated monomer was retained, indicating virtually no protein degradation with almost a five $\log_{10}$ titer reduction of the suspended PPV.

A New Tool

Because relatively inexpensive equipment can be used to produce PUV light (248 nm) capable of handling large volumes with operational ease, this viral inactivation technique is cost effective for relevant industries. The procedure has the potential to be combined synergistically with other inactivation methods. PUV laser light offers a new, nonadditive and chemically safe alternative for the treatment of FBS to inactivate adventitious virus and to preserve the biological activity necessary for the propagation of cell culture. ***BPI***

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West Nile Virus

Information on West Nile virus is available on the following websites.

- CDC Divison of Vector-Borne Infectious Diseases www.cdc.gov/ ncidod/dvbid/westnile/
- Also at CDC: questions and answers about West Nile virus: www.cdc.gov /ncidod/dvbid/westnile/q&a.htm
- U.S. Geological Survey: West Nile Virus Maps 2002: cindi.usgs.gov/ hazard/event/west_nile/west_nile.html
- National Biological Information Infrastructure: westnilevirus.nbii.gov
- West Nile Virus Surveillance Information – Bureau of Infectious Diseas – Health Canada: www.hc-sc.gc.ca/pphb-dgspsp/wnv-vwn