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EVALUATION OF HEPATITIS B VIRUS PHOTOINACTIVATION IN SERUM AND CELLULAR BLOOD COMPONENTS BY THE POLYMERASE CHAIN REACTION

Fabrizio Saraceni

A Thesis

Submitted to the Graduate College of Bowling Green State University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 1992

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ABSTRACT

Donald W. Deters, Advisor

The purpose of this study was to investigate the usefulness of the polymerase chain reaction as a tool in the photoinactivation of transfusion transmitted viruses. There are currently two major methods of inactivating viruses in blood components. One is an oxygen dependent, membrane directed method and the other is a nucleic acid directed method. Current methods of evaluating photoinactivation involve either viral cultures or chimpanzee infectivity studies. These evaluation methods require from one week to one year to obtain results. The polymerase chain reaction amplifies a region of the viral deoxyribonucleic acid by repeated denaturing-annealing-extending of that region. If viral deoxynucleic acid is inactivated by crosslinking in the nucleic acid-directed inactivation procedure, the denaturation step can not proceed and previously positive results, using the polymerase chain reaction, will now be negative. This study used the polymerase chain reaction to evaluate the inactivation of hepatitis B virus with psoralen compounds in a nucleic acid-directed procedure. We have found that under conditions which allow inactivation of the hepatitis virus by psoralens, the polymerase chain reaction could quantify the inactivation, within one or

two days, with a sensitivity comparable to chimpanzee infectivity studies. These findings demonstrate the usefulness of the polymerase chain reaction in the investigation of viral photoinactivation.

Per Papà e Mamma.

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INTRODUCTION

The transfusion of blood products is never undertaken lightly. While a transfusion can save a life, it is also recognized that it can transmit diseases. Donor screening and increased testing of blood products has greatly improved the safety of the blood supply. Yet, no test is one hundred percent perfect and there is no test for the disease that has yet to be discovered. This situation occurred with the human immunodeficiency virus (HIV). This virus entered the blood supply undetected because until March 1, 1985, there was no licensed screening test available. Now that there is a test, the transmission of HIV through the blood supply has been greatly reduced.

HIV is not the only disease that can be transmitted through the blood supply. The transmission of hepatitis via the blood supply is of much more concern than the transmission of HIV. Current estimates suggest that the risk of transfusion-transmitted HIV infection ranges from 1 in 40,000 to 1 in 150,000 donations,[1,2] and that the rate for hepatitis C virus (HCV) infection is approximately 1 in 1000 after the introduction of testing for anti-HCV.[3,4] The risk of hepatitis B virus (HBV) infection is very low, with about 100 clinically apparent, acute cases reported annually from about 6 million donations.[5]

While testing for anti-HCV has reduced the transmission of non-A, non-B

hepatitis, there is still a significant rate of transmission of non-A, non-B, non-C hepatitis. This virus (which may be called hepatitis F virus[6]) lacks a screening test, and may be transmitted by more than one virus. In addition there are other disease-causing organisms that could potentially be present in the blood supply. For example, recently over 500,000 potential donors were deferred when it was discovered that seven soldiers who had been stationed in Southwest Asia contracted *Leishmania tropica* in a mild systemic form. Since leishmaniasis can be transmitted through blood transfusions and there is no screening test for it, the Assistant Secretary of Defense for Health Affairs directed that blood donations from all personnel who were in Southwest Asia since 1 August 1990 be deferred indefinitely.[7]

As each new transfusion-transmitted disease is discovered, either a new test is added to the growing list of screening tests or a segment of the donor population is deferred. Currently the list of tests required includes alanine aminotransferase level, a test for the detection of hepatitis B surface antigen (HBsAg), antibodies to human T-lymphocyte virus I/II (anti-HTLV I/II), antibodies to human immunodeficiency virus 1 (anti-HIV-1), antibodies to hepatitis C virus (anti-HCV), antibodies to hepatitis B core (anti-HBc) and a serological test for syphilis.[8] This not only adds to the cost of processing a unit of blood components, but also reduces the amount of blood components that is available for safe transfusions. During World War II, the need for large quantities of plasma products stimulated the development of ultraviolet (UV) light treatments to inactivate the infectious agent of serum hepatitis. Still, cases of

hepatitis attributable to irradiated plasma and concerns about inactivation of serum components led to the abandonment of UV sterilization.[9] Lack of reproducible results, use of different wavelengths, inaccurate lamp calibrations and other factors tend to cast some doubt about the validity of abandoning UV treatment.

Recently, the interest in viral inactivation has increased. Several papers have been published describing various methods to inactivate viruses. These methods employ visible light (400-700 nm) and photodynamic agents,[10-15] or ultraviolet light (<400 nm) with[15-18] and without photodynamic agents.[9] It is postulated that each of the these methods inactivate viruses by different mechanisms. In a paper by Dodd, Moroff, and Wagner[15] a comparison of two different viral inactivation methods were made and it was found that under certain conditions, viruses can be inactivated and platelet function retained.

The study by Dodd, Moroff, and Wagner explored the advantages and disadvantages of the two major methods of inactivating blood components currently being investigated. This study compared an oxygen-dependent, membrane directed method using merocyanine 540 (MC540) and visible light to a nucleic acid-directed procedure using aminomethyl-trimethylpsoralen (AMT) with ultraviolet light (UVA, 320-380 nm). It was found that both methods could inactivate 5-6 $\log_{10}$ of vesicular stomatitis virus (VSV) and other model viruses in platelet suspensions with reduced plasma concentration. However, the MC540 method showed considerable damage to

platelet function when compared to the AMT-UVA method.

Photochemical inactivation or photolysis of any molecule begins by excitation of an electron from its ground state to an excited state, which leads to a chemical reaction. Irradiation of a virus with UV light induces photochemical modifications in both its nucleic acid and protein components.[19] In the case of nucleic acids, the spectrum of modifications includes formation of photodimers, photohydrates and other adducts,[20] a sufficient number of which produce a lethal 'hit' if unrepaired. Accumulation of a certain critical number of 'hits' in a viral DNA or RNA will overcome repair processes and lead to failure of that genome to replicate.[19] The larger the genome (or target) the greater the likelihood of accumulating this critical number in the same molecule or set of molecules, so that larger viruses are killed more easily.[9]

Photodynamic agents which are activated by visible light are thought to inactivate viruses by the formation of singlet oxygen.[21] Singlet oxygen production is the result of an energy transfer process following activation of the photodynamic agent via the absorption of light using a wavelength within the electronic absorption spectrum of the compound.[22] It is thought that these agents preferentially bind to the membrane of lipid-enveloped viruses and when activated, the resultant singlet oxygen produces both membrane and nucleic acid damage.[23,24]

Inactivation using photodynamic agents and ultraviolet light begins with the reversible intercalation (figure 1) of the photodynamic agent into DNA and RNA helices.[25] UV irradiation of an intercalated photodynamic molecule causes the covalent addition of this molecule to pyrimidine bases in the nucleic acid.[26,27] If the photodynamic molecule is intercalated between opposite and adjacent pyrimidine bases, a covalent crosslink may be formed between the two nucleic acid strands.[28] Crosslinked DNA and RNA helices, in the absence of repair, are essentially impossible to transcribe or replicate accurately and completely.[29-31] Viruses or cells which become heavily crosslinked by these photodynamic agents are unable to replicate. If the extent of this crosslinking approaches one photodynamic molecule per hundred base pairs, the expression of any single gene becomes impossible.[32] The study by Dodd, Moroff and Wagner indicates that the DNA directed approach provides the most promise. In particular, the psoralen family of photoreactive chemicals appear to have certain properties which make them preferable to use as photochemical inactivators. Besides their ability to crosslink nucleic acids, the psoralens are favored because their toxicity is known from their use in treatments for psoriasis and vitiligo. Therefore the concentration used for viral inactivation is within human toxicity levels.

Several psoralen derivatives have been studied as potential photoinactivators for blood components (figure 2). Naturally occurring psoralens, such as 5- and 8-methoxypsoralen, are found in buttercups, carrot greens, celery, clover, cockleburs,

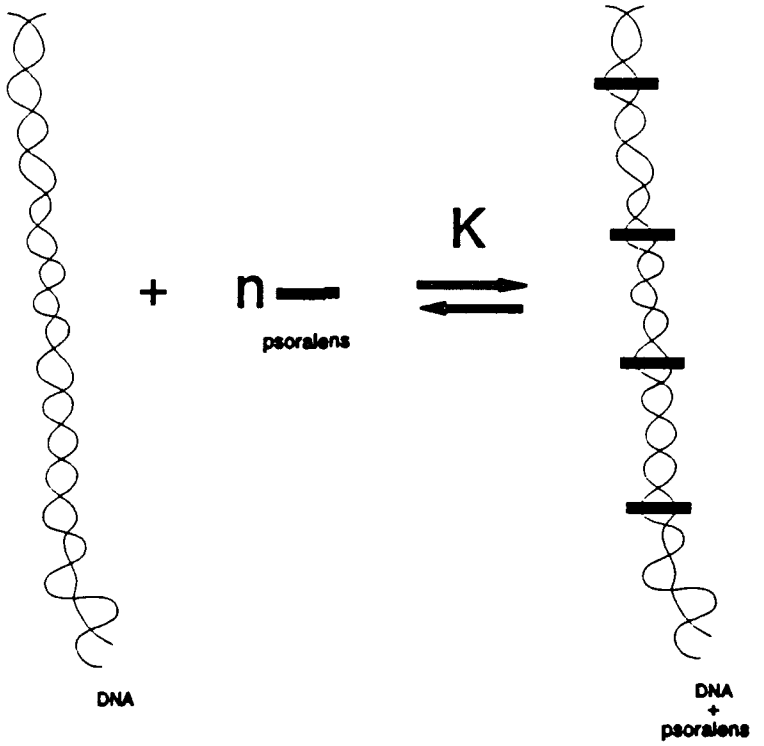


Figure 1. Intercalation of psoralens and double stranded DNA. **K** is the dissociation constant for intercalation.

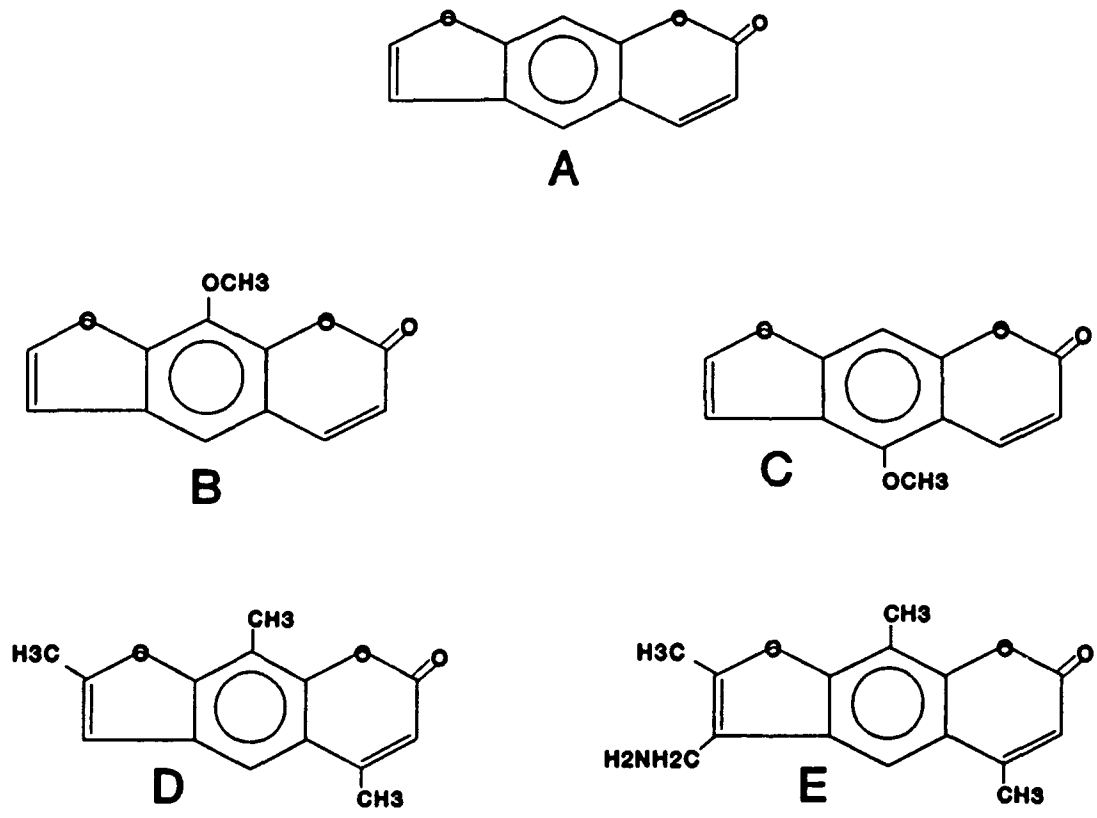


Figure 2. Chemical structures of psoralen compounds: (A) Psoralen; (B) 8-Methoxypsoralen; (C) 5-Methoxypsoralen; (D) 4,5',8-Trimethylpsoralen; (E) 4'-Aminomethyl-4,5',8-trimethylpsoralen.

dill, figs, limes, parsley, and meadow grass. Some psoralens, such as 8-methoxypsoralen and 4,5',8-trimethylpsoralen, are produced as pharmaceuticals to enhance skin pigmentation. Several new psoralen derivatives were synthesized by Isaacs, Shen, Hearst, and Rapoport[33] that had superior crosslinking abilities over 8-methoxypsoralen and 4,5',8-trimethylpsoralen. The psoralen derivative which demonstrated the greatest ability at crosslinking nucleic acids and had the greatest solubility in water was 4'-aminomethyl-4,5',8-trimethylpsoralen.

Many useful applications have been found for psoralens as molecular probes because of their photoreactivity with nucleic acids. It is thought that once an intercalated psoralen molecule is exposed to UVA light, it can form covalent bonds with nearby pyrimidine bases. There are two possible types of electronic excitation of psoralens in the ultraviolet and near-ultraviolet (200-400 nm) region of the spectrum. In one type, the non-bonding electron in the carbonyl group can be excited to the antibonding π molecular orbital (π^*) to produce the n, π^* excited state. The other type is the excitation of a π-electron to the antibonding π^*, which gives a π,π^* excited state.

It is thought that psoralens only photoreact with pyrimidine bases. Several studies have indicated that either the 3,4- or the 4',5'-carbon-carbon double bond of psoralen adds across the 5,6-carbon-carbon double bond of the pyrimidine base to give a 3,4- or 4',5'-furocoumaryl-pyrimidine cycloadduct. The photoadducts are

photodissociable upon reirradiation with 254 nm ultraviolet light, indicating that they are probably of the cyclobutane type found in thymine dimers.

Several methods have been used to evaluate the effectiveness of viral inactivation procedures. These include measuring plaque forming colonies in cell cultures,[9-11,13,14] neutral red dye uptake assay[12,15] and chimpanzee studies.[16] Most of the studies into photoinactivation have used model virus systems to measure the effectiveness of the inactivation procedure. These viruses include feline leukemia virus (FeLV), vesicular stomatitis virus (VSV), and encephalomyocarditis virus (EMCV). Only a relative few studies have used HIV or HBV in their studies. Studies which have employed HIV have evaluated its inactivation by using viral cell cultures to propagate the virus and then measuring its reverse transcriptase activity. Other methods of evaluation include measurement of cell forming colonies and red dye uptake, which require from three to twelve days to complete. The only study to directly measure hepatitis B virus inactivation evaluated the effectiveness through chimpanzee studies. Chimpanzee studies require inoculation of chimpanzees with inactivated serum and then monitoring the chimpanzees for development of the viral disease. This could take one year or more. The chimpanzees are monitored during this time period and serological tests are performed to detect an active hepatitis infection.

An alternative method would be to analyze the viral DNA for damage with

polymerase chain reaction (PCR) technology. The PCR is an *in vitro* method for amplifying a specific DNA sequence. Two oligonucleotide primers that hybridize to opposite strands of DNA are selected and mixed with an extract of the desired DNA. A thermostable DNA polymerase from *Thermus aquaticus* (*Taq* polymerase) is introduced. This mixture then undergoes a series of thermal denaturation-annealing-extension cycles where the amount of the selected DNA sequence is doubled after each cycle. In this case, the larger the region that is framed by these primers, the greater the likelihood that crosslinking will occur within that region. If crosslinking occurs within this region, amplification could not occur since the two strands of DNA would be unable to denature sufficiently to allow the primers and polymerase to insert and continue with the amplification cycles. In this way, successful inactivation could be detected by the inability of the PCR to amplify the viral DNA of interest.

In a recent study by Ulrich, Bhat, Seto, *et al.*,[34] the sensitivity and specificity of PCR for the detection of HBV was investigated. These researchers found that when analyzing serum samples with known quantities of chimpanzee infectious doses (CID_{50}) of HBV, PCR was more sensitive than chimpanzee studies. A chimpanzee infectious dose is defined as the amount of infectious material contained in one milliliter of serum which can induce infectivity in $\geq$50% of inoculated chimpanzees. The samples studied contained 10^7 and $10^{7.5}$ CID_{50} and could be detected by PCR at dilutions of up to 10^{-8}, levels below the limit of infectivity.

Several questions need to be answered before PCR can be used to evaluate viral photoinactivation. Since *Taq* polymerase has some 5' to 3' exonuclease activity during polymerization, there is the possibility that damaged DNA, which had in fact been inactivated, could be repaired during the thermal cycles. This would lead to amplification of DNA which was inactivated successfully. Also, would the cyclobutane structure of the crosslinks withstand the thermal cycling of the PCR? If the cyclobutane structure is dissociated by the heat of the PCR reaction, successfully inactivated DNA would be amplified as if it were intact. This study will attempt to answer these questions by analyzing samples which have been photoinactivated using PCR.

MATERIALS AND METHODS

Tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetic acid buffer

All DNA samples were diluted or reconstituted with a 10 mM tris(hydroxymethyl)aminomethane (Tris)/1 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.5) buffer. This buffer was prepared by adding one volume of a stock solution of 1 M Tris/0.1 M EDTA (pH 7.5) (Biofluids, Rockville, MD) to ninety nine volumes of RNase and pyrogen free diethylpyrocarbonate (DEPC) treated water (Biotecx Laboratories, Houston, TX). This buffer will be referred to as TE buffer.

Proteinase K digestion buffer

Proteinase K digestion buffer was used for the extraction of DNA from platelet suspensions. This buffer was prepared by adding 625 μl of 1 M tris-HCl (pH 7.5) (Biofluids, Rockville, MD), 625 μl of a 10% sodium dodecyl sulfate solution (SDS) (BRL, Gaithersburg, MD), 5 μl of 0.5 M EDTA (pH 8.0) (NIH Media Unit, Bethesda, MD) and 2.5 mg of proteinase K (Boehringer-Mannheim, Germany) to a quantity of DEPC treated water sufficient to make a final volume of 10 ml. This buffer will be referred to as PK buffer.

Hepatitis B virus serum

Sera containing Hepatitis B virus (HBV) was obtained from known positive stock samples maintained at the Transfusion Transmitted Viruses Laboratory, Department of Transfusion Medicine, Warren Grant Magnuson Clinical Center, National Institutes of Health, Bethesda, Maryland. These samples had been tested for Hepatitis B surface antigen (HBsAg), using a radioimmunoassay procedure (Ausria, Abbott Laboratories, Chicago, Illinois) and the sample with the highest concentration of Hepatitis B surface antigen was chosen for this study. One serum sample, identified as 311982 (subtype *ad*, 138 μg/ml HBsAg) was chosen for this study.

Human platelets and platelet suspensions

Platelets were obtained from the Dowling Apheresis Center of the Department of Transfusion Medicine, Warren Grant Magnuson Clinical Center, National Institutes of Health, Bethesda, Maryland. The platelets were collected by apheresis using the Baxter-Fenwall CS3000 plus. The platelets were used after expiration and contained the equivalent of six to nine units of single donor platelets in a reduced volume. In order to simulate the equivalent concentration of platelets in a single donor platelet unit, platelet suspensions were made by diluting one volume of the apheresis platelet samples with five volumes of TE buffer to make a 1:6 dilution. In order to make HBV positive platelet suspensions, one volume of HBV serum was added to every

1000 volumes of platelet suspension.

Extraction of viral DNA from serum

A 20 μl portion of serum was incubated in a sterile 1.5 ml microcentrifuge tube (PGC Scientifics, Gaithersburg, MD) with 20 μl of 0.2 N sodium hydroxide (NaOH) (Sigma) at 37°C for 60 minutes. The solution was neutralized with 20 μl of 0.2 N hydrochloric acid (HCl) (Sigma) and 60 μl of water, then centrifuged at 5000 rpm for five minutes. The supernatant (100 μl) was transferred with a Gilson Pipetteman pipettor using disposable pipette tips (PGC Scientifics) to another sterile 1.5 ml microcentrifuge tube and used as the DNA template taking care not to disturb the pellet.

Extraction of viral DNA from platelet suspensions

A 100 μl sample of platelet suspension was incubated in a sterile 1.5 ml microcentrifuge tube with 400 μl of PK buffer at 65°C for 2 hours. This gave a final concentration of 0.2 mg/ml proteinase K, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 0.5% SDS when added to the aliquot of platelet suspension. After incubation, an equal volume of redistilled, nucleic acid grade phenol (BRL, Gaithersburg, MD) was pipetted to the platelet suspension with PK buffer and vortexed for 15 seconds. The microcentrifuge tube was then centrifuged at 5000 g for 10 minutes at 5°C to separate

the two layers. The aqueous layer was transferred to another microcentrifuge tube with a Gilson Pipetteman pipettor using disposable tips. An equal volume of phenol-chloroform (J.T. Baker, Phillipsburg, NJ)-isoamyl alcohol (Sigma, St. Louis, MO) mixture (25:24:1 ratio) was added to the aqueous layer, vortexed for 15 seconds and then centrifuged at 5000 g for 5 minutes at 5°C. The aqueous layer was transferred to another microcentrifuge tube and an equal volume of chloroform- isoamyl alcohol (24:1) was added. This mixture was vortexed for 15 seconds and then centrifuged at 5000 g for 5 minutes at 5°C. The aqueous layer was then precipitated in 1000 μl of 100% ethanol (Midwest Grain Products, Weston, MO) containing 50 μl of 3 M sodium acetate (pH 8.0) (NIH Media Unit, Bethesda, MD) and 10 μl of a 1 mg/ml solution of yeast tRNA (Boehringer-Mannheim, Germany) at -20°C for 2 hours followed by centrifugation at 14,000 g for 15 minutes at 0°C. The ethanol was decanted and the centrifuge tube was allowed to air dry for 30 minutes. The pellet was then resuspended in TE buffer and incubated at 37°C for 30 minutes. This sample was used as the DNA template.

Light sources

Ultraviolet-C light was provided by an irradiator (UV Stratalinker 1800, Stratagene, La Jolla, CA) using five 254 nm germicidal fluorescent bulbs (eight watts, NIS G8T5 GL-8). The amount of light delivered was determined by using the Stratalinker in the energy mode. In the energy mode, the amount of

microjoules/centimeter^2 is programmed into the Stratalinker and the lights will turn off when the appropriate amount of light energy has been delivered.

Low intensity ultraviolet-A light was provided by an irradiator (UV Stratalinker 1800, Stratagene, La Jolla, CA) using five 365 nm blacklight fluorescent bulbs (eight watts, NIS F8T5BL FL8BL) in the energy mode.

High intensity ultraviolet-A light was provided by an irradiator (Derma Control, Frankfort, IL) at the Jerome H. Holland Laboratory, American Red Cross , Rockville, MD, courtesy of Dr. Roger Dodd and Dr. Steve Wagner. This irradiator contained two banks of six tanning bulbs (F587T12BLH0, Derma Control) and was placed on a flatbed agitator (Melco Engineering). The amount of light was measured using a light meter (International Light Meter, Model IL1400A, Newburyport, MA) with a ultraviolet-A light detector (International Light Meter, Model SEL033). The amount of light was measured in milliwatts/centimeter^2 and converted to millijoules/centimeter^2 using the following formula: (milliwatts/centimeter^2) x (seconds) = millijoules/centimeter^2. The light intensity from each bank of lights was measured and then summed. This sum was then divided by the desired energy to be delivered to the samples to determine the amount of time that the samples were to be irradiated.

Psoralens

4,5',8-Trimethylpsoralen (TMP) (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (Sigma, St. Louis, MO) at a concentration of 5 mg/ml. This stock solution was stored at -20°C.

4'-Aminomethyl-4,5',8-trimethylpsoralen (AMT) (HRI Associates, Concorde, CA) was generously provided by Dr. Dodd and Dr. Wagner. It was dissolved in distilled water at a concentration of 4.36 mg/ml. This stock solution was stored at room temperature.

Irradiation of samples

For low intensity irradiation, a 1 ml aliquot of the desired sample was placed in one well (35 mm diameter) of a six well tissue culture dish (Costar, Cambridge, MA) and then placed into the Stratalinker irradiator. The light path for these samples was 1 mm. The appropriate energy level was selected and the aliquot irradiated.

For high intensity irradiation, a 4 ml aliquot of platelet suspension was placed in a 60 mm diameter petri dish (Fisher Scientific, Pittsburgh, PA) and then placed into the high intensity irradiator for the appropriate amount of time to achieve the desired energy level. The light path for these samples was 1.4 mm.

PCR amplification of HBV DNA

A 10 μl aliquot of the DNA extracted using the sodium hydroxide procedure or a 20 μl aliquot of the DNA extracted with proteinase K was amplified in a 100 μl final volume reaction mixture containing 2 units of recombinant *Taq* DNA polymerase (AmpliTaq; Perkin-Elmer Cetus, Norwalk, Conn.), 40 μM concentration of each deoxynucleotide triphosphate (dNTP) (Perkin-Elmer): deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP) and deoxycytidine triphosphate (dCTP), a 0.2 μM concentration of each primer (Nucleic Acid and Protein Synthesis Laboratory, Fredrick Cancer Research Facility, National Cancer Institute, Fredrick, MD) 50 mM KCl, 10 mM Tris HCl (pH 8.3) and 1.5 mM $MgCl_2$. The reaction mixture was placed into a 0.5 ml reaction tube (GeneAmp, Perkin-Elmer, Branchburg, NJ) and overlaid with 50 μl of mineral oil (Sigma). Primer 1C 5'-GGGTGGAGCCCTCAGGCTCAGGGCA-3' (25-mer) and primer 2C 5'-GAAGATGAGGCATAGCAGCAGGAT-3' (24-mer) were used. The amplification by this pair begins at position 1679 (1C) and ends at position 2254 (2C) of the HBV genome resulting in a 575 base pair fragment as the PCR product.[35]

The reaction was performed for 35 cycles in a programmable thermal cycler (Perkin-Elmer Cetus). Samples were heated to 94°C for 30 seconds (denaturation of DNA), cooled to 55°C for 60 seconds (annealing to primer), and incubated at 72°C for 60 seconds (polymerase amplification reaction). After completion of all 35

cycles, the samples were cooled to 10°C to stop the PCR. All experiments were performed in parallel with positive and negative controls. Negative controls were prepared from TE buffer, treated as serum samples from the DNA extraction step through the PCR amplification.

This PCR product was then used as a template for a second PCR. In this second PCR, a 10 μl aliquot of product from the first PCR was added to a PCR reaction mixture containing 2 units of recombinant *Taq* DNA polymerase (AmpliTaq; Perkin-Elmer Cetus, Norwalk, Conn.), 40 μM of each deoxynucleotide triphosphate (dNTP): deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP) and deoxycytidine triphosphate (dCTP), a 0.2 μM concentration of each primer, 50 mM KCl, 10 mM Tris HCl (pH 8.3) and 1.5 mM $MgCl_2$ in a final volume of 100 μl. The reaction mixture was placed into a 0.5 ml reaction tube and overlaid with 50 μl of mineral oil. Primer 1A 5'-CTCCTGCCTCCACCAATC-3' (18-mer) and primer 2A 5'-GGGGACTGCGAATTTTGG-3' (18-mer) were used. The amplification by this pair begins at position 1731 (1A) and ends at position 2125 (2A) of the HBV genome resulting in a 411 base pair fragment as the final PCR product.

Preparation of agarose gel

The agarose gels used in analyzing the amplified DNA were prepared by

adding 1.2 grams of agarose (BRL, Gaithersburg, MD) to 100 ml of 0.04 M Tris-HCl/0.0083 M sodium acetate/0.0011 M EDTA buffer (pH 7.8) (Biofluids, Rockville, MD) and microwave heating for 2 minutes on high to dissolve the agarose. The 1.2% agarose was placed on a stirring plate and 100 μl of a stock 10 mg/ml solution of ethidium bromide (Sigma) was added. After a few minutes, the agarose was poured into a 10 cm by 15 cm tray with a 15 or 20 well template. The gel was allowed to harden for thirty minutes before use.

Analysis of amplified DNA

After final amplification, 10 μl of dye was added to the PCR reaction tube. This dye contains 20% ficoll (Sigma), 0.1% xylene cyanole (United States Biochemical Corporation, Cleveland, OH), and 0.1% bromophenol blue (Pharmacia LKB, Piscataway, NJ) in 100 ml of distilled water. A 20 μl sample of dyed, amplified DNA reaction mixture was then loaded onto the agarose gel and electrophoresed at 100 volts for one hour (BioRad Electrophoresis Apparatus Model 200, BioRad, Richmond, CA) in 0.04 M Tris-HCl/0.0083 M sodium acetate/0.0011 M EDTA buffer (pH 7.8). When electrophoresis was completed DNA in the gel was visualized by placing the gel on a UV transilluminator (UVP Inc., San Gabriel, CA) emitting 254 nm light. The bands were then recorded on film using a Polaroid MP4 Land Camera (Polaroid, Cambridge, MA) with orange and yellow filters, Polaroid type 57 high-speed (3000 ASA) black and white film, f/16 aperture setting, and

shutter speed one second.

Experimental studies

UVC effects on HBV DNA. One milliliter of HBV DNA extracted from serum 311982 using sodium hydroxide was placed into a well of a six well tissue culture plate and irradiated with 0, 60, 120, 180, 240, 300, and 360 mjoules of 254 nm low intensity light. The sample was then serially diluted by pipetting 10 μl of the sample and adding it to a 1.5 ml microcentrifuge tube containing 90 μl of TE buffer, making a 1:10 dilution. Then 10 μl of this 1:10 dilution was added to another microcentrifuge tube containing 90 μl TE buffer, using a clean pipette tip, making a 1:100 dilution. These steps were repeated until a final dilution of 1:10,000 was achieved. Each diluted sample was then used as a source of DNA for the PCR using primers 1C and 2C. In this experiment only the initial PCR was performed.

Platelet effects on UVC inactivation. Apheresis platelets were diluted 1:6 with TE buffer and then HBV serum was added so that the serum was diluted 1:100. One ml of this sample was placed into a well of a six well tissue culture plate and irradiated with 900 mjoules of 254 nm low intensity light. Another aliquot was not exposed to light. The samples were then serially diluted by pipetting 10 μl of each sample and adding it to a 1.5 ml microcentrifuge tube containing 90 μl of TE buffer, making a 1:1000 dilution of the original HBV concentration. Then 10 μl of this

1:1000 dilution was added to another microcentrifuge tube containing 90 μl TE buffer, using a clean pipette tip, making a 1:10,000 dilution. These steps were repeated until a final dilution of 1:10,000,000,000 was achieved. Each diluted sample was then used as a source of DNA for the PCR using primers 1C and 2C. The PCR products from the first PCR were used as templates for the second PCR using primers 1A and 2A.

Irradiation of platelet suspensions with TMP. Apheresis platelets were diluted 1:6 with TE buffer and then HBV serum was added so that the serum was diluted 1:1000. One ml of this sample was placed into two wells of a six well tissue culture plate and 10 μl of the stock solution of TMP was added to one well, so that the final concentration was 50 μg/ml of TMP. One ml of diluted apheresis platelets, without HBV, was added to two additional wells of the six well tissue culture plate and 10 μl of the stock TMP solution was added to one of these wells. The samples were allowed to set for 20 minutes to enable the TMP to intercalate with the viral DNA. Then the tissue culture plate was irradiated with 2.5 joules of 365 nm low intensity light. An additional 10 μl of stock TMP solution was added to the wells which contained TMP initially and the plate was irradiated for an additional 2.5 joules. Duplicate platelet suspensions were not exposed to light. The viral DNA was extracted from the samples using the proteinase K buffer and amplified using the PCR procedure with primers 1C and 2C. The PCR products from the first PCR were then used as templates for a second PCR using primers 1A and 2A.

Irradiation of diluted HBV serum with TMP. HBV serum was added to TE buffer so that the serum was diluted 1:1000. One ml of this sample was placed into three wells of a six well tissue culture plate and 10 μl of the stock solution of TMP was added to two wells, so that the final concentration was 50 μg/ml of TMP. A sample of the diluted HBV serum was treated with TMP, but not exposed to light. The samples were allowed to set for 20 minutes to enable the TMP to intercalate with the viral DNA. Then the tissue culture plate was irradiated with 2.5 joules of 365 nm low intensity light. One sample containing TMP was removed and an additional 10 μl of stock TMP solution was added to the remaining well which contained TMP initially. The plate was irradiated with an additional 2.5 joules. The viral DNA was extracted from the samples using the proteinase K buffer and amplified using the PCR procedure with primers 1C and 2C. The PCR products from the first PCR were then used as templates for a second PCR using primers 1A and 2A.

Irradiation of platelet suspensions with AMT. Apheresis platelets were diluted 1:6 with TE buffer and then HBV serum was added so that the serum was diluted 1:1000. Four milliliters of this sample was placed into six petri dishes and 37 μl of the stock solution of AMT was added to three dishes, so that the final concentration was 40 μg/mL of AMT. The samples were allowed to set for 20 minutes to enable the AMT to intercalate with the viral DNA. Then two samples, one containing AMT, the other without, were irradiated with 2.4 joules of 365 nm high intensity light. Another pair of samples, one with AMT, were irradiated with

4.8 joules. The last pair of samples were irradiated with 7.2 joules. Duplicate platelet suspensions were not exposed to light. The viral DNA was extracted from the samples using the proteinase K buffer. The DNA extract from each sample was serially diluted 1:100 to a final dilution of 1:100,000,000,000 of the original HBV serum sample and amplified using the PCR procedure with primers 1C and 2C. The PCR products from the first PCR were then used as templates for a second PCR using primers 1A and 2A.

Standardization of HBV serum. HBV serum 311982 was standardized by comparing its highest detectable titer by PCR with that obtained from sera with known quantities of HBV subtype *adw* and *ayw*. The infectivities of the initial specimens were calculated to be $10^{7.0}$ (*adw*) and $10^{7.5}$ (*ayw*) CID_{50}.[36] Each sample was serially diluted by pipetting 10 μl of the sample to a 1.5 ml microcentrifuge tube containing 90 μl of TE buffer, making a 1:10 dilution of the initial HBV concentration. Additional serial ten-fold dilutions were made until a final dilution of $1:10^{10}$. Each diluted sample, beginning with the 10^{-5} dilution through to the 10^{-10} dilution, was used as a source of DNA for the PCR using primers 1C and 2C. The PCR products from the first PCR were used as templates for the second PCR using primers 1A and 2A.

Dosage effects. Apheresis platelets were diluted 1:6 with TE buffer and then HBV serum was added so that the serum was diluted 1:100. Four milliliters of this

sample was placed into six petri dishes and 37 μl of the stock solution of AMT was added to three dishes, so that the final concentration was 40 μg/ml of AMT. The samples were allowed to set for 20 minutes to enable the AMT to intercalate with the viral DNA. Then two samples, one containing AMT, the other without, were irradiated with 0.24 joules of 365 nm high intensity light. Another pair of samples, one with AMT, were irradiated with 0.48 joules. The next pair of samples were irradiated with 0.97 joules. The last pair of samples were irradiated with 1.94 joules. Duplicate platelet suspensions were not exposed to light. The viral DNA was extracted from the samples using the proteinase K buffer. The DNA extract from each sample was serially diluted 1:10 to a final dilution of 1:100,000,000 of the original HBV serum sample and amplified using the PCR procedure with primers 1C and 2C. The PCR products from the first PCR were then used as templates for a second PCR using primers 1A and 2A.

RESULTS

UVC effects on HBV DNA

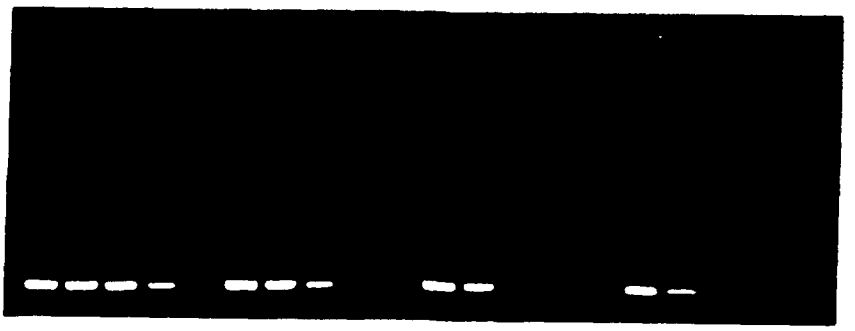
The purpose of this study was to examine the feasibility of using PCR to detect crosslinking caused by UVC light in HBV DNA diluted in TE buffer. Using the single PCR technique, the nonirradiated HBV DNA samples tested were detected up to a 1:10,000 dilution. When samples were exposed to various amounts of UVC light, titered and then amplified with PCR, it was found that with increasing UV energy the highest detectible titer decreased, however, at 0.36 joules the undiluted sample could not be amplified (Figure 3). This indicated that crosslinking caused by the UVC light had occurred within the 579 base pair sequence framed by the oligoprimers used in this experiment and this crosslinking was able to maintain its integrity during the rigors of the PCR.

Platelet effects on UVC inactivation

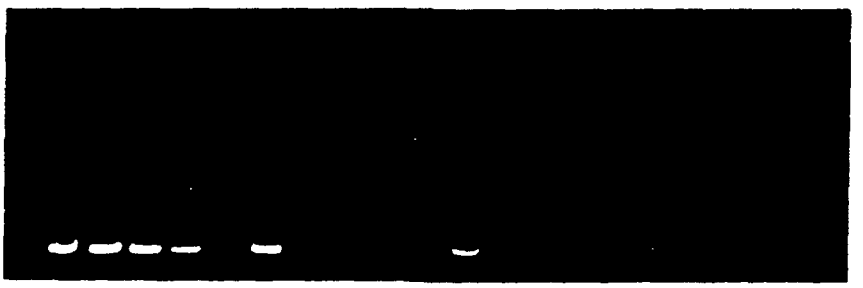
In this study, we looked at the effect platelets would have on viral inactivation. In the pilot study, 0.36 joules of UVC light was sufficient to inactivate HBV DNA in a buffer solution. When HBV serum is added to a 1:6 dilution of apheresis platelets (for a final dilution of 1:100 HBV) and exposed to UVA light, only at 0.9 joules did we begin to observe a 2 $\log_{10}$ decrease in the highest detectable titer (Figure 4). This

Figure 3. UVC effects on HBV DNA

Sample A was not irradiated, sample B was irradiated with 0.06 joules, sample C was irradiated with 0.12 joules, sample D was irradiated with 0.18 joules, sample E was irradiated with 0.24 joules, sample F was irradiated with 0.30 joules and sample G was irradiated with 0.36 joules. Each sample was serially diluted ten-fold to a final dilution of $1:10^3$ after exposure to UVC light. Supercripts refer to the dilution of HBV relative to the stock HBV serum.



$A^0A^{-1}A^{-2}A^{-3}$ $B^0B^{-1}B^{-2}B^{-3}$ $C^0C^{-1}C^{-2}C^{-3}$ $D^0D^{-1}D^{-2}D^{-3}$

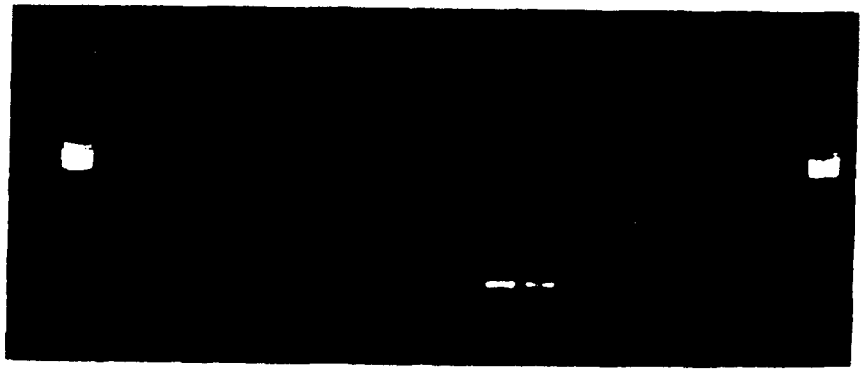


$A^0A^{-1}A^{-2}A^{-3}$ $E^0E^{-1}E^{-2}E^{-3}$ $F^0F^{-1}F^{-2}F^{-3}$ $G^0G^{-1}G^{-2}G^{-3}$

Figure 4. Platelet effects on UVC inactivation

In figure 4A, the highest detectable titer for the 1:100 HBV dilution in the platelet suspension is $1:10^6$. In figure 4B, the highest detectable titer for the sample exposed to 0.9 joules is $1:10^4$. The platelet count for the diluted platelets was 159,000/μl. M is the 123 base pair ladder marker and 0 is the reagent blank. Lower case letters identify the products from the first PCR, upper case letters identify the products from the second PCR. Superscripts refer to the dilution of HBV relative to the stock HBV serum.

M 0 a^{-2} a^{-3} a^{-4} a^{-5} a^{-6} a^{-7} a^{-8} a^{-9} 0 A^{-2} A^{-3} A^{-4} A^{-5} A^{-6} A^{-7} A^{-8} A^{-9} M



M 0 b^{-2} b^{-3} b^{-4} b^{-5} b^{-6} b^{-7} b^{-8} b^{-9} 0 B^{-2} B^{-3} B^{-4} B^{-5} B^{-6} B^{-7} B^{-8} B^{-9} M

indicated that platelets, and other components present in a unit of platelets, absorb a sufficient amount of UVC light to provide some protection against damage to the HBV DNA.

Irradiation of platelet suspensions with TMP

In this study, we explored the feasibility of measuring the effects of TMP on HBV DNA using PCR. When samples of 1:6 dilution of apheresis platelets with HBV (1:1000) were exposed to 365 nm low intensity light, there was no reduction of the highest detectable titer between samples exposed to light with 50 μg/ml of TMP added and those samples which did not have TMP added (figure 5). This indicated that either the TMP was not effective as a photoinactivating agent or that PCR was unable to distinguish the products of the photoreaction between TMP and HBV DNA from unmodified DNA.

Irradiation of diluted HBV serum with TMP

In order to resolve the results of the previous study, we measured the effects of TMP on HBV DNA in the absence of platelets. When HBV serum was diluted (1:1000) and exposed to UVA light (low intensity), there was a discernable difference in the fluorescence intensity of the ethidium bromide stained HBV DNA which was exposed to UVA light and the HBV DNA which was not exposed to UVA light

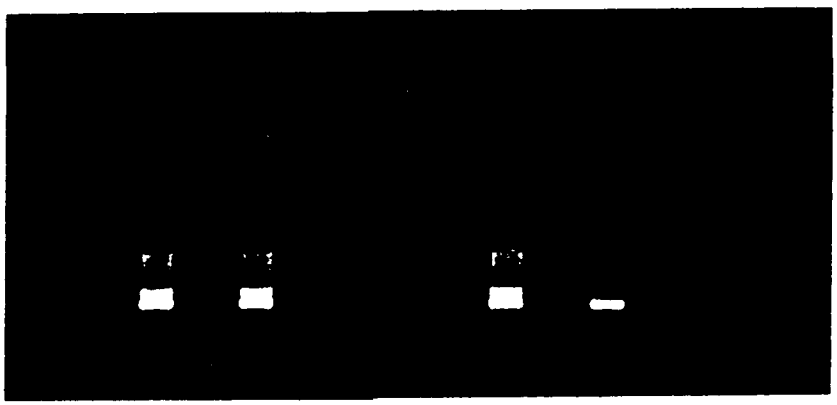


Figure 5. Irradiation of platelet suspensions with TMP

Slot A is a sample of diluted platelets with HBV added, not exposed to UVA light. Slot B is a sample of diluted platelets that was not exposed to light. Slot C is a sample of diluted platelets with HBV and TMP (50 μg/ml) added that was not exposed to light. Slot D is a sample of diluted platelets with TMP added, not exposed to light. Slot 0 was the reagent blank. Slot 3A is a sample of diluted platelets with HBV added, exposed to UVA light for a total of 5 joules. Slot 3B is a sample of diluted platelets that was exposed to light. Slot 3C is a sample of diluted platelets with HBV and TMP (50 μg/ml) added that was exposed to light. Slot 3D is a sample of diluted platelets with TMP added, exposed to light.

(figure 6). However, there was no difference between the UVA exposed samples which had TMP added (50 μg/ml) and those which did not have TMP. These results indicated that the amount of UVA light used (5 joules) was sufficient to damage the DNA when platelets were not present and TMP was not effective when platelets were present.

Irradiation of platelet suspensions with AMT

In this study, we used a psoralen derivative which had a greater solubility in water and a greater affinity for DNA along with a more intense light source. We found that 2.4 joules of UVA light was sufficient to damage the DNA in the samples which contained the AMT so that PCR could not amplify the DNA which was present (figure 7). The samples which did not have AMT added showed no effect on the highest detectable titer up to the highest energy level used. This indicated that PCR could be used to detect damage to HBV DNA caused by UVA light and AMT.

Standardization of HBV serum

In order to quantitate the sensitivity of our PCR method, we measured the highest detectable titer of our HBV serum sample and compared the results to the highest detectable titer of two sera which were standardized via chimpanzee studies. The two sera, *adw* and *ayw*, contained 10^7 chimpanzee infective doses (CID_{50}) and

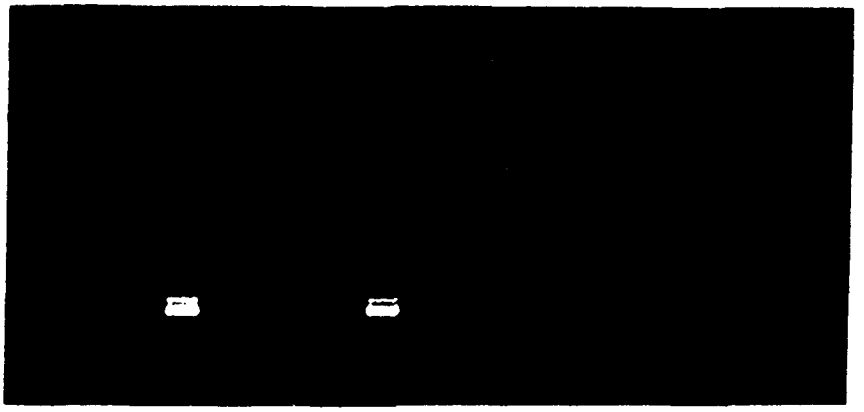


Figure 6. Irradiation of diluted HBV serum with TMP

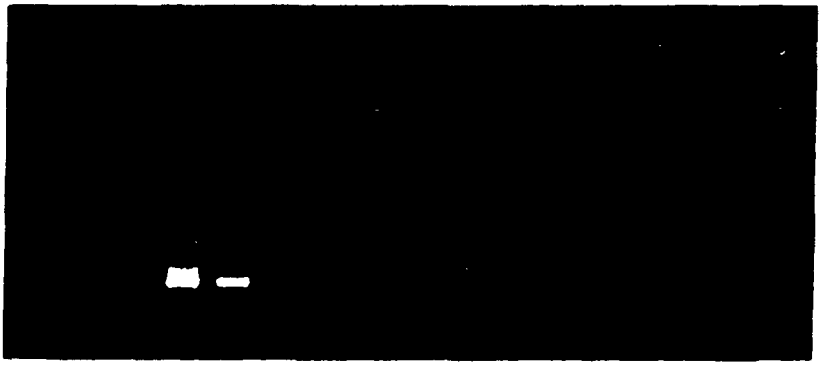
In figure 6, slot A0 is a sample of diluted HBV not exposed to UVA light. Slot A5 is a sample of diluted HBV that was exposed to light (5 joules). Slot B0 is a sample of diluted HBV and TMP (50 μg/ml) added that was not exposed to light. Slot B2.5 is a sample of diluted HBV with TMP added, exposed to light for 2.5 joules. Slot B5 is a sample of diluted HBV with TMP added, exposed to UVA light for a total of 5 joules. Slot 0 is the reagent blank. As can be seen from figure 6, there is no difference between slot A0 and B0.

Figure 7. Irradiation of platelet suspensions with AMT

Samples A, C, E and G were diluted apheresis platelets (1:6) with a final platelet count of 289,000/μl. HBV serum was added to these samples for an initial dilution of 1:1000. Samples B, D, F, and H were identical to A, C, E and G except AMT was added at a final concentration of 40 μg/ml. Samples A and B were not exposed to high intensity UVA light. Samples C and D were exposed to 2.4 joules of high intensity UVA light. Samples E and F were exposed to 4.8 joules of high intensity UVA light. Samples G and H were exposed to 7.2 joules of high intensity UVA light. Sample 0 was the reagent blank. Superscripts refer to the dilution of HBV DNA relative to the stock HBV serum.

A^{-3} A^{-5} A^{-7} A^{-9} A^{-11} B^{-3} B^{-5} B^{-7} B^{-9} B^{-11} C^{-3} C^{-5} C^{-7} C^{-9} C^{-11}

D^{-3} D^{-5} D^{-7} D^{-9} D^{-11} E^{-3} E^{-5} E^{-7} E^{-9} E^{-11} F^{-3} F^{-5} F^{-7} F^{-9} F^{-11}



0 G^{-3} G^{-5} G^{-7} G^{-9} G^{-11} H^{-3} H^{-5} H^{-7} H^{-9} H^{-11}

$10^{7.5}$ CID_{50} respectively. The highest detectable titer for the two standard sera was $1:10^7$, while the highest detectable titer for our HBV serum was $1:10^8$ (figure 8). This showed that our PCR titers correlated well with chimpanzee infective doses and that our HBV serum had a greater titer than the standard sera.

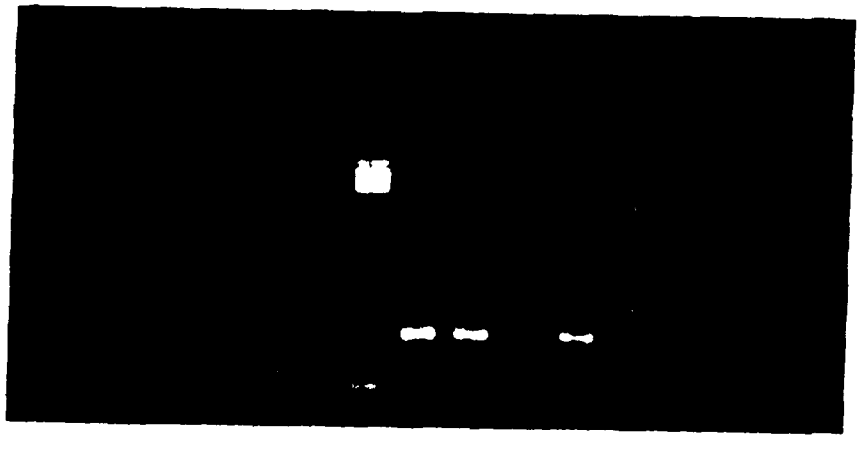
Dosage effects

We applied our PCR method to the measurement of the effects that various doses of UVA have on HBV DNA in platelet suspensions when AMT was added and when AMT was not added. We found that there was no difference between the highest detectable titer of the samples which did not contain AMT, regardless of the amount of UVA light the samples were exposed to, up to 14.4 joules. There was also no difference in highest detectable titer between the two samples which were not exposed to light, whether AMT was present or not. When a sample containing AMT was irradiated with 0.24 joules (10 seconds), there was a 2 $\log_{10}$ decrease in the highest detectable titer, compared to the unirradiated sample. At 0.48 joules, there was a 4 $\log_{10}$ decrease; at 0.97 joules a 6 $\log_{10}$ decrease; and at 1.94 joules the HBV DNA could not be amplified (figure 9). This showed that every doubling of the amount of light exposure resulted in a 2 $\log_{10}$ decrease in the highest detectable titer.

Figure 8. Standardization of HBV serum

The highest detectable titer, as measured by PCR, for HBV serum samples *adw* and *ayw* was $1:10^7$. HBV serum sample *adw* (sample D) was calculated to contain 10^7 CID_{50} and HBV serum sample *ayw* (sample Y) was calculated to contain $10^{7.5}$ CID_{50}. HBV serum sample 311982 (sample a and sample A) was detectable up to a $1:10^8$ dilution. Lower case letter refers to product from first PCR, upper case letter refers to product from second PCR. Superscripts refer to the dilution of the serum.

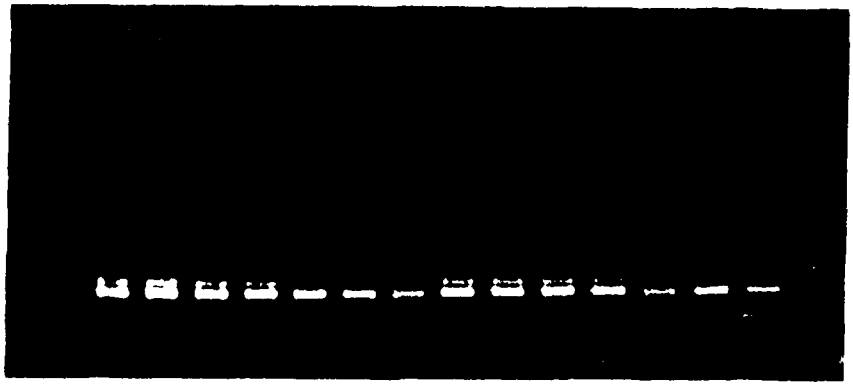
D^{-5} D^{-6} D^{-7} D^{-8} D^{-9} D^{-10} Y^{-5} Y^{-6} Y^{-7} Y^{-8} Y^{-9} Y^{-10}



a^{-5} a^{-6} a^{-7} a^{-8} a^{-9} a^{-10} M A^{-5} A^{-6} A^{-7} A^{-8} A^{-9} A^{-10} 0

Figure 9. Dosage effects

All samples initially contained a 1:6 dilution of apheresis platelets (441,000 platelets/μl) and a 1:100 dilution of HBV serum. Samples B-F contained 40 μg/ml of AMT. Samples A and B were not exposed to light. Sample C received 0.24 joules of UVA light. Sample D received 0.48 joules of UVA light. Sample E received 0.97 joules of UVA light. Sample F received 1.94 joules of UVA light. Sample 0 was the reagent blank. Each sample was serially diluted after treatment. Superscripts refer to the dilution of HBV serum relative to the stock HBV serum.



0 A^{-2} A^{-3} A^{-4} A^{-5} A^{-6} A^{-7} A^{-8} B^{-2} B^{-3} B^{-4} B^{-5} B^{-6} B^{-7} B^{-8}

C^{-2} C^{-3} C^{-4} C^{-5} C^{-6} C^{-7} C^{-8} D^{-2} D^{-3} D^{-4} D^{-5} D^{-6} D^{-7} D^{-8}

E^{-2} E^{-3} E^{-4} E^{-5} E^{-6} E^{-7} E^{-8} F^{-2} F^{-3} F^{-4} F^{-5} F^{-6} F^{-7} F^{-8}

DISCUSSION

The intent of this study was to see if PCR could detect irreparable damage to the HBV genome caused by irradiation with UV light and therefore be used as a tool to evaluate HBV photoinactivation. By forming photodimers, photohydrates and other adducts the UV light causes the HBV DNA to crosslink in such a way that it may not be able to denature when heated to 94°C. If the DNA cannot denature, the primers cannot anneal to the DNA and amplification will not occur. Viruses treated in such a way are effectively inactivated since they cannot replicate.

In order to be assured that the PCR cannot proceed because of DNA crosslinking, one needs to be certain that the conditions for the PCR are optimized so amplification of a specific DNA sequence can be consistently reproduced. Several factors influence the extent of amplification and the specificity of the DNA products of the PCR.

The most critical factor is the primer pair. Unfortunately, there is as yet no specific rule to follow in the selection of efficient and specific primers. There are a few general guidelines that help in the selection of primers.[37] Primers should be selected with a random base distribution and with a guanosine/cytidine (GC) content similar to that of the fragment being amplified. It is best to avoid sequences with significant secondary structures, particularly at the 3'-end of the primer and to avoid

primers with 3' overlaps. Most primers will be between 20 and 30 bases in length and the optimal amount to use in an amplification will vary. Primer concentrations between 0.1 and 0.5 μM are generally optimal. Higher primer concentrations may promote mispriming and accumulation of nonspecific products and may increase the probability of generating a template-independent artifact termed a primer-dimer. These artifacts compete with the desired product for enzyme, dNTPs, and primer resulting in a lower yield of the desired product.[38]

The concentration of $MgCl_2$ in the PCR reaction buffer can have a profound effect on the specificity and yield of an amplification. Generally, excess Mg^{2+} will result in the accumulation of non-specific amplification products and insufficient Mg^{2+} will reduce yield.[37,38]

The deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP) are usually present at concentrations of 50 to 200 μM each. Higher concentrations may tend to promote misincorporations by the polymerase and should be avoided.[38] At 50 to 200 μM, there is sufficient precursor to synthesize approximately 6.5 to 25 μg of DNA. Deoxynucleotide concentrations between 20 and 200 μM each result in the optimal balance among yield, specificity and fidelity. The four dNTPs should be used at equivalent concentrations to minimize misincorporation errors. One should decide on the lowest dNTP concentration appropriate for the length and composition of the target sequence since specificity and fidelity are increased by using lower dNTP

concentrations. Low dNTP concentrations minimize mispriming at non-target sites and reduce the likelihood of extending misincorporated nucleotides.

The concentration of *Taq* polymerase typically used in PCR is about 2.5 units per 100 μl reaction. For amplification reactions involving DNA samples with high sequence complexity, such as genomic DNA, there is an optimum concentration of enzyme, usually 1 to 4 units per 100 μL. Increasing the amount of enzyme beyond this level can result in greater production of non-specific PCR products and reduced yield of the desired target fragment.[38]

PCR is performed by incubating the samples at three temperatures corresponding to the three steps in a cycle of amplification-denaturation, annealing, and extension. This cycling was accomplished automatically with the DNA Thermal Cycler (Perkin-Elmer Cetus Instrumentation). In a typical reaction, the double stranded DNA is denatured by briefly heating a sample to 90-95°C, the primers are allowed to anneal to their complementary sequences by briefly cooling to 40-60°C, followed by heating to 70-75°C to extend the annealed primers with the *Taq* polymerase. The time of incubation at 70-75°C varies according to the length of the target sequence being amplified. A good starting point is one minute for every kilobase of sequence, shortening the time once other amplification conditions have been established.[37]

Insufficient heating during the denaturation step is a common cause of failure in a PCR reaction. It is important that the reaction reaches a temperature at which complete strand separation occurs. A temperature of about 94°C is usually sufficient and was selected for this experiment. As soon as the sample reaches 94°C, it can be cooled to the annealing temperature. Extensive denaturation is unnecessary and limited exposure to elevated temperatures helps maintain maximum polymerase activity throughout the reaction.[37]

The temperature at which the annealing is done depends on the length and GC content of the primers. A temperature of 55°C is a good starting point for typical 20-base oligonucleotide primers with about 50% GC content; even higher temperatures may be necessary to increase primer specificity. Because of the very large molar excess of primers present in the reaction mixture, hybridization occurs almost instantaneously and long incubation at the annealing temperature is not needed.[38]

Extension time depends upon the length and concentration of the target sequence and upon temperature. Primer extensions are traditionally performed at 72°C. Estimates for the rate of nucleotide incorporation at 72°C vary from 36 to 100 nucleotides per second depending upon the buffer, pH, salt concentration and the nature of the DNA template. An extension time of 60 seconds at 72°C is considered sufficient for products up to 2000 bases in length.[37,38]

The amplification reaction is not infinite. After a certain number of cycles the desired amplification fragment gradually stops accumulating exponentially and enters a linear or stationary phase. This second stage of the reaction is called the "plateau." The point at which a PCR reaction reaches its plateau depends primarily on the number of copies of target originally present in the sample and by the total amount of DNA synthesized. In addition to exhaustion of primer or dNTP or inactivation of polymerase or dNTP there are three other causes of plateau: substrate excess conditions, competition by non-specific products, and product reassociation[37].

Once PCR conditions were optimized, we began to evaluate the photoinactivation of HBV. The first study was to see if the effects of 254 nm light could be measured and quantitated using PCR. As seen in figure 3, there was a measurable difference in the highest detectable titer of HBV DNA samples exposed to UVC light prior to amplification with PCR. This effect was quantitated by titration of the sample before amplifying. The effects of UVC light are cumulative so that the greater the amount of light energy absorbed by the HBV DNA, the greater the amount of damage.

Our PCR at this point could only detect a $1:10^4$ dilution of the initial HBV serum sample. Studies using chimpanzees to measure the infectivity of HBV in serum have reported titers of 10^7 to 10^8 CID_{50}.[34,36] To enhance the sensitivity, without using radioactive probes, we decided to use nested primers in an additional PCR on

the first PCR product. We examined the sensitivity of this procedure by serially diluting our stock HBV serum and amplifying the titrations with the PCR using primers 1C and 2C. Afterwards, we used the amplified product as a DNA template for a second PCR using primers 1A and 2A. The principal purpose of nesting is to overcome the plateau phenomena by using primers to a region within the amplified segment of the original DNA target. Using nested primers we were able to detect HBV DNA in our sample up to a $1:10^8$ dilution (figure 8). When this procedure was applied to serum samples with known amounts of CID_{50}, both samples, *adw* and *ayw*, could be amplified up to a dilution of $1:10^7$. Sample *adw* contained 10^7 CID_{50} and sample *ayw* contained $10^{7.5}$ CID_{50} as measured by the National Center for Biologics Evaluation and Research using chimpanzee studies. This indicated that our method was as sensitive as chimpanzee studies and that our sample contained 10^8 CID_{50}. With this enhanced sensitivity we could easily detect a 6 $\log_{10}$ reduction in viral titer.

When we added platelets to our HBV serum, we noticed a marked increase in the amount of UVC light required before any effect could be detected (figure 4). The main chromophore during UVC irradiation appears to be DNA.[39] However, when proteins and cellular material are also present, as in blood products, they act as additional chromophores to absorb a portion of the UV light, requiring an increase in the amount of UV light to see an effect on DNA. Light scatter is another effect produced by cellular material when present, necessitating an increase in light energy to see inactivation.

The main chromophore during UVA irradiation does not appear to be DNA, but rather proteins.[39] During UVC irradiation the major damage to DNA is caused by the formation of cyclobutyl pyrimidine dimers, leading to crosslinking. However, during UVA irradiation the ratio of cyclobutyl pyrimidine dimers formed to single strand breaks is reduced. This indicates that while the formation of cyclobutyl pyrimidine dimers is the major product of UVC irradiation, single strand breaks are the major product of UVA irradiation. In a biological system, single strand breaks are more easily repaired than cyclobutyl pyrimidine dimers are excised, but DNA excision repair can remove these dimers if they are not too numerous.

The amount of UVC light needed to inactivate viruses in blood components is also enough to cause cellular damage to the components themselves.[15] UVA light does not cause as much cellular damage as UVC light, but is not as efficient as UVC light in inactivating viruses.[39] Psoralen compounds can form cyclobutyl adducts when exposed to UVA light in the presence of DNA. This mimics the cyclobutyl pyrimidine dimers formed when DNA is exposed to UVC light. In this way, one can have the virucidal effects of UVC without the accompanying cellular damage.

When we exposed our platelet suspensions, with HBV and TMP added, to UVA light, we could not detect a significant decrease in the amplified PCR product (figure 5). This caused some concern since TMP and UVA light had been used by Alter[16] in inactivating blood components. In Alter's study, chimpanzees were

inoculated with the inactivated samples and monitored for development of hepatitis. The chimpanzees did not develop hepatitis from these inoculations indicating that the inactivation procedure was effective. The procedure that was used irradiated a 10 ml sample for 18 hours, adding additional TMP every hour. In the study by Dodd[15], 4 ml samples containing model viruses only required 2 minutes of UVA radiation to inactivate the viruses. However, Dodd used AMT as the photochemical agent. Isaacs[33] studied the properties of various psoralen compounds. He found that the solubility of TMP in water was 0.6 μg/ml, while the solubility of AMT was 10^4 μg/ml. The dissociation constant (K_D) for the noncovalent binding of each psoralen to DNA was 5.6×10^{-5} mol/l for TMP and 6.6×10^{-6} mol/l for AMT. This indicated that AMT had an affinity for DNA that was ten times greater than TMP. In combination with its greater solubility in aqueous solutions, Isaacs calculated that the ratio of psoralen bound sites to unbound sites in DNA was 5000 for AMT versus 0.046 for TMP. This led us to the possibility that TMP was not effective at inactivating HBV under our conditions. There was also the possibility that the PCR procedure was repairing the dimers, through the 5' to 3' exonuclease activity of the *Taq* polymerase.[40]

In an attempt to resolve these questions, we irradiated a diluted sample of HBV serum with TMP. In the absence of platelets and the accompanying proteins, 5 joules of UVA light not only inactivated the HBV when TMP was present, but it was sufficient to damage the HBV DNA without TMP also (figure 6). This did not

answer the question about the effectiveness of TMP since we could not determine from this data if the effect observed in the TMP containing samples was due to the UVA alone or UVA and TMP in combination. However, 5 joules of UVA light was sufficient to detectably damage the HBV DNA. This demonstrated the inhibitory effect that proteins have on viral photoinactivation.[15] Dodd found it necessary to remove at least 85% of proteins in a unit of platelets before inactivation could be successful. For this reason we diluted our apheresis platelets 1:6.

In order to investigate photoinactivation of HBV with photochemical agents, we needed an alternative to TMP. We chose to use AMT since it had been reported to be 5000 times more efficient at intercalating DNA than TMP.[33] When AMT was used as the photoactive agent, HBV was inactivated with only 2.4 joules of UVA light (figure 7). Samples containing HBV and platelets, but no AMT were not inactivated. This indicated that the platelets and protein present protected the HBV and that the photochemical formation of cyclobutyl dimers by AMT with DNA could withstand the rigors of the PCR.

In measuring the effects of various amounts of UVA light on platelet suspensions with a fixed amount of AMT, we could see that as the amount of light increased, the amount of detectable DNA decreased (figure 9). This clearly demonstrated that we could quantitate the increasing damage to the HBV DNA by using PCR.

In order to test the effectiveness of inactivation of HBV, various methods have been used. Most of these methods have involved viral culture techniques. However, in vivo infectivity testing conducted in chimpanzees has been found to be the most sensitive method for detecting HBV in serum. Therefore, infectivity testing in chimpanzees has been used to verify the safety of plasma fractions and hepatitis B vaccines derived from human blood. However, all the aforementioned methods require days[11-15,17] (viral cultures) or months[16] (chimpanzee studies) before results are available.

PCR can be performed and analyzed within hours. We have demonstrated that the sensitivity of our PCR method is comparable to chimpanzee infectivity studies. PCR was able to amplify HBV DNA at a dilution of 10^{-8}. Therefore, if PCR cannot detect HBV DNA in serum, it most likely does not contain infectious HBV. Then, if a sample which does contain infectious HBV is treated with UV light and subsequently PCR can no longer amplify the HBV DNA, the HBV has been inactivated.

This study shows that PCR is an effective tool in the analysis of HBV photoinactivation. PCR is comparable to chimpanzee infectivity studies and takes less time than other methods of analysis currently in use. This will allow more rapid development of an effective method of inactivating HBV and other transfusion transmitted viruses. At this time, UV inactivation appears to have the most promise.

By combining inactivation procedures with PCR analysis perhaps a truly practical, efficient and economical sterilization method may be developed sooner.

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