PHOTOCHEMICAL DECONTAMINATION OF BLOOD COMPONENTS CONTAINING HEPATITIS B AND NON-A, NON-B VIRUS

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Summary Diluted plasma samples containing 10^2, 10^3, 10^4, and 10^5 chimpanzee infectious doses (CID) of a human non-A, non-B hepatitis virus (NANBV) were treated with a combination of two psoralen compounds, 4'-aminomethyl-4,5',8-trimethylpsoralen and 4,5',8-trimethylpsoralen, and exposed to long wavelength ultraviolet. Each dilution was then transfused into one of four chimpanzees. In a second experiment, three samples containing $10^{4.5}$ CID of hepatitis B virus (HBV) and two samples containing 10^4 CID of NANBV were treated with 8-methoxypsoralen (8-MOP) and ultraviolet irradiation. Two chimpanzees were each transfused with both a treated HBV and a treated NANBV sample. The third chimpanzee was inoculated with a treated HBV sample alone. In the six months after inoculation none of the animals showed biochemical or histological evidence of hepatitis. In experiments involving NANBV inocula, the susceptibility of the animals was confirmed by subsequent challenge with untreated NANBV. Factor VIII concentrate containing virus and photochemically treated as in the first experiment retained an average of 91% of its activity while that in the second experiment retained 94% of its activity.

Introduction

THE safety of the blood supply continues to be threatened by the transmission of viral agents, particularly non-A, non-B hepatitis virus (NANBV), human immunodeficiency virus (HIV), and hepatitis B virus (HBV).[1] NANB hepatitis (NANBH) develops in 5–10% of multiply transfused recipients, accounting fo 150 000 to 300 000 cases annually in the USA.[2] Although sensitive serological assays exist for HIV and HBV, these agents can still occasionally be transmitted by seronegative blood donors.[3,4] Up to 10% of tranfusion-related hepatitis and 25% of severe, icteric cases are due to HBV transmitted by hepatitis B surface antigen (HBsAg) negative donors.[3] 13 cases of transfusion-related HIV infections have been reported to the Centers for Disease Control among recipients of blood pretested for anti-HIV.[4] Among haemophilic patients receiving plasma or clotting factor concentrates, up to 90% have serological evidence of past HBV or HIV infection, and many have overt hepatitis B or AIDS; 45–80% of haemophiliacs have a raised serum transaminase in the absence of HBsAg, suggesting chronic NANBH.[5]

Because the increasing number of direct and indirect donor screening measures required to protect the blood supply, the most promising approach to the reduction of transfusion-associated disease is the biophysical removal or biochemical inactivation of hepatitis and other blood transmitted viruses. We report here some experimental

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findings with a photochemical decontamination (PCD) technique, psoralen plus long wave ultraviolet light, that offers a potential means of preserving the biological functions of proteins and cells while destroying viral reproductive capability.[6,7]

Methods

General Study Design

The study was performed in two phases. In phase I, dilutions of a human NANBV inoculum containing 10^2 to 10^5 CID_{50}/ml (CID = chimpanzee infectious dose) were treated under code with a combination of two psoralen compounds, 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT) and 4,5',8-trimethylpsoralen (TMP) (HRI Associates, Emeryville, CA), and inoculated into four chimpanzees. When the chimpanzees were seen not to get hepatitis, the animal receiving the highest titred inoculum was challenged with untreated NANBV-containing plasma to test its susceptibility.

In phase II of the study, a third psoralen, 8-methoxypsoralen (8-MOP) (Aldrich, Inc, Milwaukee, WI), was used alone because it was a Food and Drug Administration licensed product previously administered to human subjects. Additionally, we addressed the efficacy of photochemical decontamination for the inactivation of HBV and assessed whether inactivation could be achieved in factor VIII concentrates as well as in plasma or phosphate-buffered saline (PBS).

Light Source

Samples were irradiated from above and below by General Electric type F20T12-BLB fluorescent UVA bulbs with an electric fan blowing gently across the lights to cool the area. Available UVA was measured with both light banks on by a Black-Ray Long Wave UV meter (Ultraviolet Products, Inc, San Gabriel, CA). The total measured intensity was 7·4 mW/cm².

Blood Products

Fetal calf serum (FCS) (CGS, KC Biological, Lenexa, KS) was heat-inactivated at 56°C before use. Solutions of bovine serum albumin (BSA) (Sigma Chemical Co, St Louis, MO) were prepared in PBS (pH 7·4). Factor VIII (AHF) concentrates ('Koate', Cutter Biological, Berkeley, CA) were obtained as lyophilised material and stored at 4°C until used; for use, each vial was reconstituted with 10 ml sterile distilled water.

'patitis Virus Inocula

The NANBV inocula obtained from human plasma (Hutchinson, H strain) with an infectivity titre of 10^6 CID_{50}/ml,[9] were stored in various dilutions at −80°C. For these experiments, dilutions ranging from 10^{-1} to 10^{-5}, containing 10^5, 10^4, 10^3, and 10^2 CID/ml, were used. In phase I, these samples were coded to ensure blind evaluation of the inactivation procedure, frozen, and stored at −80°C until use. In phase II the same NANBV inoculum was given, at a 10^{-1} dilution (10^5 CID). The phase II experiment was unblinded.

The HBV inoculum (titre $10^{4.5}$ CID_{50}/ml) consisted of the MS-2 *ayw* strain diluted in FCS and was kindly supplied by Dr Saul Krugman and Dr Robert Purcell. Both the NANBV and HBV inocula have been extensively characterised and represent the standard challenge inocula for hepatitis experimentation in chimpanzees.

Challenge Inocula

Challenge in phase I was confined to a single animal (D) that received an inoculum containing 10^5 CID NANBV which had been inactivated. The challenge sample for this animal was 10 ml of a 1:33 000 dilution of the original 10^{-1} dilution of NANBV nominally containing 33 CID. The challenge was administered 28 weeks after the test dose. Animals in phase II that received treated NANBV were challenged at 42 weeks with 10^4 CID of untreated NANBV. Challenges with untreated HBV were not performed because HBV susceptibility could be established with sensitive serological markers.

Preparation and Inactivation of NANBV in Phase I Study

The coded samples of NANBV were thawed rapidly and 0·9 ml of each was added to 9·1 ml of a solution containing 1% BSA, 5 mmol sodium ascorbate (Sigma), 20 μg/ml AMT, and 0·5 μg/ml TMP. Each diluted solution was placed in a BSA-rinsed T-75 polystyrene tissue culture flask (Corning, Corning, NY) and incubated in the dark for 10 h at ambient temperature. The head space in each flask was flushed with humidified argon gas for 1 h and the flask was re-sealed before exposure to the UVA. Every hour for 13 h, the flasks were removed from the light source and 20 μg/ml AMT and 0·5 μg/ml TMP was added to them. They were flushed again with argon and replaced under the lights. The solutions were transferred to fresh BSA-rinsed flasks at every third drug addition. The four samples were then frozen for later inoculation into chimpanzees A–D.

Preparation and Inactivation of NANBV and HBV in Phase II Study

Each of three animals in the phase II experiment received an inoculum containing either HBV and NANBV or HBV alone. In each case, these inocula were treated with 8-MOP followed by UVA irradiation for 10 h. Animal E received $10^{4.5}$ CID HBV diluted in 1 ml FCS, 0·92 ml NANBV containing 10^4 CID, 300 μg/ml 8-MOP, 2·6 ml PBS, 0·1 ml R17 phage, and 6 μl heparin. A second animal, F, received a similar inoculum except that 2·6 ml of reconstituted factor VIII concentrate replaced the PBS as the suspending medium. A third animal, G, received $10^{4.5}$ CID HBV in 1 ml FCS, 300 μg/ml 8-MOP, 3·6 ml PBS, and 0·1 ml R17. The bacteriophage R17 was used as an internal control to validate the PCD treatment. Each solution was placed in a silanised medicine bottle fitted with a rubber septum. The head space in each flask was flushed for 1 h with humidified argon gas before exposure to UVA. After 5 h of UVA irradiation, the solutions were placed in a second bottle, flushed again, and returned for 5 more hours of UVA exposure. After inactivation, the samples were frozen for shipment and subsequent inoculation into the three chimpanzees (E–G).

Chimpanzee Model

All chimpanzees were housed at the Southwest Foundation for Biomedical Research. Chimpanzees were housed in climate controlled isolation buildings in individual cages which did not allow for physical contact or exchange of body fluids between animals. Animals used in the NANBH experiments (phase I and II) had never been experimentally exposed to NANBV. They had previously been used in HBV vaccine safety studies, but remained negative for HBsAg, anti-HBc, and anti-HBs. Animals observed in the hepatitis B experiments (phase II) had not previously been experimentally exposed to HBV and were negative for HBsAg, anti-HBc, and anti-HBs.

Clinical Follow-up and Laboratory Evaluation of Inoculated Chimpanzees

Complete delivery of the inacuvated or challenge inocula was ensured by intravenous administration and by flushing of the syringe with recipient blood. In each chimpanzee, blood samples were obtained each week beginning four weeks before inoculation, on the day of inoculation, and then weekly for six months after inoculation. Each sample was tested for alanine aminotransferase (ALT). Samples from HBV inoculated animals were also tested weekly for HBsAg ('Austria', Abbott Laboratories, Chicago IL) and monthly for anti-HBc and anti-HBs ('Corab' and 'AusAb', Abbott).

Closed liver biopsy was done before inoculation and then routinely at weeks 5, 7, 9, 11, 13, 20, and 26 post inoculation. Additional biopsy samples were obtained as indicated by ALT levels greater than 40 IU/L. Liver biopsy specimens were divided

such that one segment was prepared for light microscopy and one was prepared for thin-section electron microscopy. Slides for light microscopy were prepared by the American Histo-Lab Inc. (Gaithersburg, MD) and were read under code by Dr Hans Popper (Mount Sinai Hospital, New York, NY). For electron microscopy, liver tissue was fixed in glutaraldehyde, postfixed in osmium tetroxide, treated with uranyl acetate, dehydrated, infiltrated with propylene oxide, and embedded in 'Epon 812'. Thin section electron micrographs were read under code.

Baseline ALT levels were established for each animal and the normal ALT for that animal was established by determining the mean of 10 baseline values. Subsequent ALT values greater than three standard deviations above the mean (99% confidence limits) were considered abnormal. An animal was diagnosed as having hepatitis if there were two consecutive abnormal ALT values and if these abnormalities coincided with histological evidence of hepatocellular inflammation. In experiments involving the NANB agent, the finding of cytoplasmic tubular structures[9] was considered additional evidence for the diagnosis of viral hepatitis.

The diagnosis of hepatitis B depended on the de novo appearance of HBsAg and/or antibody seroconversion to the hepatitis B surface and/or core antigens. NANB hepatitis was diagnosed when biochemical and histological evidence of hepatitis occurred in the absence of serological markers for hepatitis B or hepatitis A infection ('HAVAB' and 'HAVAB-M', Abbott).

Factor VIII Samples

1·0 ml of reconstituted antihaemophilic factor (AHF) concentrate was diluted to 10 ml in the BSA/ascorbate/psoralen/PBS solution used for the NANBV experiment in phase I. A second 1·0 ml sample of the AHF was diluted in a similar solution with sterile distilled water replacing the psoralen. These samples were placed in BSA rinsed flasks, and the second flask was wrapped in foil to exclude the light (thus serving as a handling control). The samples were then processed in the same manner as the phase I NANBV samples. Sterile distilled water was added to the handling control at intervals instead of psoralen. Samples were withdrawn from each of the AHF samples every 3 h, placed in polypropylene vials, and stored at - 80°C until assayed for residual factor VIII activity.

Two parallel AHF samples were prepared for the phase II experiment; the first contained 8-MOP (300 µg/ml), 4·6 ml AHF, 0·1 ml R17 phage, and 5 µl heparin, while in the second, barbital buffer replaced the psoralen, and the bottle was wrapped in foil (the handling control). These samples were processed in the same manner as were the phase II hepatitis samples; after 10 h UVA exposure, the samples were frozen at − 80°C in polypropylene vials. Factor VIII activity in the quickly-thawed AHF samples was assayed by means of the activated partial thromboplastin time test (George King Biomedical, Kansas, KA).

Results

Toxicity

In the phase I study, a 10 ml volume of 1% BSA containing a total of 3·6 mg of AMT and 90 µg of TMP was administered intravenously to each of four chimpanzees. If we assume a total blood volume of 4 litres, this inoculum corresponds to approximately 0·9 µg/ml final serum level of AMT and 0·02 µg/ml final serum level of TMP. In the phase II study, a 10 ml volume of PBS or factor VIII concentrate containing 3 mg 8-MOP was administered intravenously to each of three chimpanzees. This corresponds to approximately 0·8 µg/ml final serum level of 8-MOP. The administration of PCD treated plasma or factor VIII did not result in any adverse reaction. The animals showed no changes in vital signs, nor was there evidence of anaphylaxis or less severe allergic reactions. During at least one year of follow-up, no long term consequences of the PCD treated plasma have been noted. All animals remain healthy, even those in which hepatitis developed on challenge. The final serum concentration of 0·8–0·92 µg/ml administered to each animal was comparable with the serum levels of psoralen reported in psoriatic patients receiving oral doses of psoralen.[10]

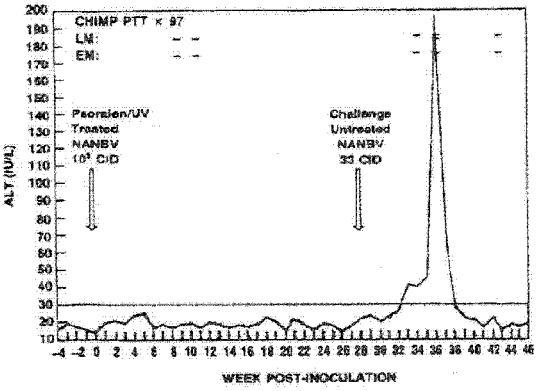

Fig 1—Serum ALT in chimpanzee D inoculated with NANBV treated with psoralen/UVA and then challenged with untreated NANBV.

LM = light microscopy; EM = electron microscopy.

Inactivation of NANBV in Phase I

Four chimpanzees were inoculated with different dilutions of the Hutchinson strain inoculum that had been treated for 18 h. None of the animals had hepatitis during 27 weeks of follow-up as assessed by transaminase measurements. Liver biopsy specimens were obtained at the appropriate times for the development of NANBH (see methods) even in the absence of a raised ALT. None showed the histological changes of viral hepatitis, and they were also devoid of structures typical of NANBV infected liver.[9] Hence, it appeared that PCD treatment had inactivated up to 10^5 CID of NANBV. To test the susceptibility of these animals to NANBV infection, and to have an animal serve as its own control, animal D, which received 10^5 CID of inactivated NANBV, was challenged with 33 CID of untreated NANBV. As seen in fig 1, whereas no evidence of hepatitis was observed for 28 weeks after receipt of PCD treated inoculum, NANBV infection developed 8 weeks after the challenge inoculum. Liver biopsy specimens revealed light microscopic changes of viral hepatitis first evident at week 7 or just before the peak transaminase activity. The histological changes were most consistent with NANBH as interpreted by Dr Popper. Cytoplasmic tubular structures (fig 2) were observed only on the 8 week post-inoculation sample coinciding with the peak ALT. The inactivation of the most potent NANBV inoculum in this series and the subsequent demonstration of NANBV susceptibility in that animal negated the need to challenge the three chimpanzees receiving inactivated virus of lower initial potency.

Inactivation of HBV and NANBV in Phase II

Three chimpanzees received HBV/NANBV-containing inocula that had been inactivated with 8-MOP for 10 h (table I). Animal E was exposed to $10^{4.5}$ CID HBV and 10^4 NANBV which had been suspended in saline before inactivation; animal F was exposed to the identical inoculum

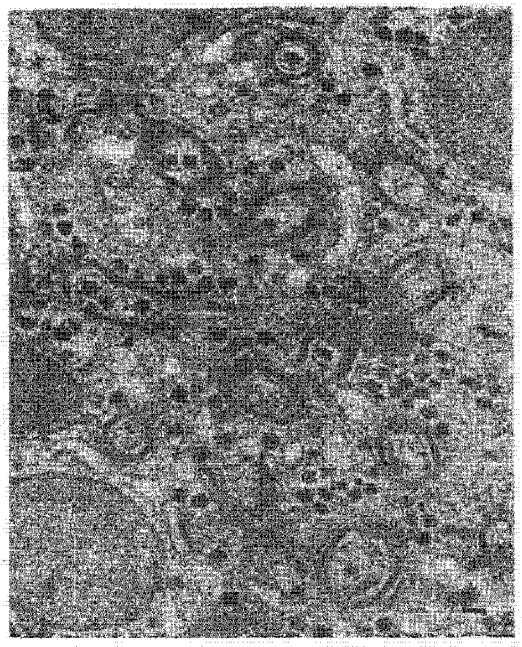

Fig 2—Transmission electron micrograph of non-A, non-B hepatitis associated cytoplasmic tubules (arrowhead) within infected chimpanzee D hepatocytes.

These tubular structures were about 300 nm in diameter and were continuous with endoplasmic reticulum membranes. Bar = 100 nm.

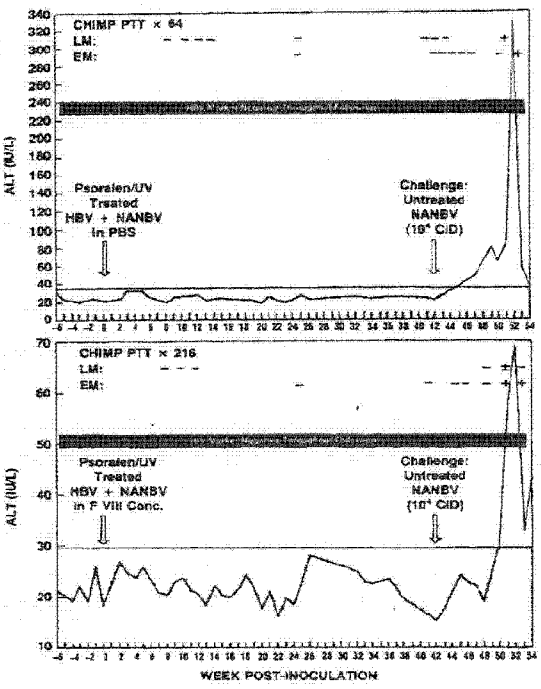

Fig 3—Serum ALT in chimpanzees E (top) and F (bottom) inoculated with HBV and NANBV treated with psoralen/UVA followed by challenge with untreated NANBV.

except that the viruses were suspended in a commercial factor VIII concentrate before photochemical decontamination. Neither animal had hepatitis type B, or hepatitis type non-A, non-B during 40 weeks of observation (fig 3). Since chimpanzees seronegative for hepatitis B markers are uniformly susceptible to challenge with $10^{4.5}$ CID of the MS-2 strain of HBV, a challenge with untreated HBV-containing material was not indicated. In contrast, animals E and F were challenged intravenously with the same NANBV inoculum (10^4 CID) which had not undergone PCD. Animal E showed clear-cut biochemical evidence of hepatitis (fig 3). A liver biopsy specimen obtained at week 10 showed acidophilic bodies, some with associated inflammatory reaction and focal necrosis. The biopsy specimen was interpreted as showing a mild hepatitis consistent with NANBH. Thin section electron microscopy demonstrated typical NANB-related cytoplasmic tubular structures.

Animal F likewise had mild hepatitis after NANBV challenge (fig 3). Light microscopy did not reveal a diagnostic histological lesion although mild portal inflammatory reaction and focal necrosis were observed. Despite the absence of definitive histological lesions of hepatitis, electron microscopy revealed cytoplasmic tubular structures in two consecutive biopsy specimens at the time of peak ALT.

In designing the study, we decided to administer treated HBV alone to one chimpanzee to exclude the possibility that hepatitis B infection might be masked by interference from coexistent NANBV infection. Hence chimpanzee G received PCD treated HBV suspended in PBS. No biochemical evidence of hepatitis was observed, and the animal did not develop HBsAg, anti-HBc, or anti-HBs. Liver histology remained normal, and ground-glass hepatocytes were not observed. This animal was not challenged with untreated HBV because of the well-documented infectivity of the MS-2 HBV inoculum.

TABLE I—PHASE II EXPERIMENT INACTIVATION OF HEPATITIS B AND NON-A, NON-B VIRUSES IN FCS-PBS AND IN FACTOR VIII CONCENTRATES

Chimpanzee	Inoculum/CID	Suspending medium	Outcome Hep B	Outcome NANBH	NANBV challenge
E	HBV, $10^{4.5}$ + NANBV 10^4	FCS-PBS	No	No	Hepatitis
F	HBV ($10^{4.5}$) + NANBV (10^4)	F VIII	No	No	Hepatitis
G	HBV ($10^{4.5}$)	PBS	No	No	Not done

TABLE II—EFFECT OF PSORALEN-UVA TREATMENT ON FACTOR VIII ACTIVITY IN AHF CONCENTRATES

PCD (h)	Factor VIII activity (U/ml) Test (PCD)	Factor VIII activity (U/ml) Handling control (no PCD)	% Factor VIII activity retained (test/control) × 100
Phase I conditions			
3	18·2	20·4	89
6	15·8	16·4	96
9	13·8	17·8	77
12	14·8	15·8	94
15	13·2	14·6	90
18	11·8	12·0	98
Phase II conditions			
10	18·8	20·2	94

Retention of Factor VIII Activity in Treated Concentrates

When AHF concentrate was treated with PCD for 18 h in the same manner as the NANBV samples in phase I, loss of factor VIII activity in the PCD treated sample was similar to loss in the handling control (table II). Loss in both samples was attributed to use of the tissue culture flasks as holding vessels because there was no loss in activity when silanised glass or plastic blood bags were used.

When AHF concentrate in silanised medicine bottles was treated with PCD for 10 h in the same manner as the HBV+NANBV samples in phase II, loss of factor VIII activity in the control and in the treated sample was small. The test sample contained 18·8 U/ml VIII activity and the handling control 20 U/ml, yielding 94% recovery of factor VIII activity in the treated sample (table II).

Discussion

In this report, we have tested photochemical decontamination, using psoralen and UVA light, as an alternative means to inactivate viruses in blood products. Psoralen derivatives bind to double-stranded nucleic acid via intercalation[11] and, in the presence of UVA, form covalent adducts to pyrimidine bases[12,13] effectively inhibiting transcription and replication.[14-16] Inactivation of enveloped and non-enveloped viruses with single or double-stranded DNA or RNA genomes has been previously shown.[17,18]

We used the chimpanzee to evaluate the ability of psoralen/UVA to inactivate both HBV and NANBV and to assess the potential for photochemicals to inactivate virus in factor VIII concentrates while retaining clotting factor activity. These experiments showed that both HBV and NANBV were sensitive to photochemical inactivation; $10^{4.5}$ CID of HBV and up to 10^5 CID of NANBV were inactivated under two separate PCD protocols. Inactivation was demonstrated by the following: the absence of biochemical or serological evidence of NANBV or HBV infection; the absence of histological evidence of viral hepatitis; and the absence of the ultrastructural changes associated with NANBV hepatitis in the chimpanzee. Challenge inocula were used to establish the susceptibility of animals used in NANBV experiments.

Two different PCD protocols were effective for inactivation of NANBV. The first PCD treatment used a combination of psoralens (TMP and AMT). TMP is a Food and Drug Administration approved psoralen derivative used for the treatment of severe psoriasis. The second PCD treatment used another derivative, 8-MOP, also used in the Psoralen + UVA (PUVA) treatment of psoriasis. Because of lack of previous experience in inactivating NANBV, very stringent PCD conditions (high psoralen concentration and long irradiation times) were used to optimise the probability of inactivating NANBV but it is possible that much less stringent PCD conditions would have been sufficient. Further experiments are needed to define the minimum effective dose of psoralen and the minimum time of UVA exposure.

In this report, we extend our earlier observations[6,7] of virus inactivation in factor VIII concentrates. It appears that both HBV and NANBV can be effectively inactivated in a solution containing factor VIII without seriously impairing clotting factor activity. In the presence of AMT and TMP, there was generally more than 90% retention of factor VIII activity even after 18 h of UVA exposure. Likewise, there was 94% retention of factor VIII activity after 10 h of 8-MOP plus UVA light. Hence, PCD appears to be a viable approach to the decontamination of factor VIII concentrates. At the present time, all such concentrates should be inactivated by heat, or with lipid solvents, or photochemically to prevent the hepatitis and AIDS risk of these products.[4,5]

Although we did not directly assess the cellular effects in the present study, PCD is not disruptive to cell membranes and has the potential to inactivate a wide range of viral agents in cellular blood products including whole blood, packed red cells, and platelet concentrates. Preliminary results with platelet concentrates have demonstrated the retention of various in vitro properties by PCD treated platelets (Lin L, Wiesehahn GP, Morel PA, Corash L, unpublished). The ability of PCD to inactivate viruses without destroying the functional integrity of the cellular elements of blood is a major potential advantage of this method compared with heat treatment or lipid solvents. Although PCD would theoretically be fully effective independent of serological testing, the combination of specific assays for HBV, HIV, and NANBV with a viral inactivation procedure could ensure that blood was no longer a vehicle for infectious disease transmission.

We thank Dr Robert H. Purcell for preparing the dilution series for NANBV; Dr Saul Krugman for providing the MS-2 strain of HBV; and Dr Lily Lin for help with preparation of the report. This work was supported by Agrion Inc and Diamond Scientific Co and a National Heart Lung and Blood Institute contract no 1-HB-7-7029.

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