Solvent/Detergent-Treated Plasma: A Virus-Inactivated Substitute for Fresh Frozen Plasma

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Fresh frozen plasma (FFP) is prepared in blood banks worldwide as a by-product of red blood cell concentrate preparation. Appropriate clinical use is for coagulation factor disorders where appropriate concentrates are unavailable and when multiple coagulation factor deficits occur such as in surgery. Viral safety depends on donor selection and screening; thus, there continues to be a small but defined risk of viral transmission comparable with that exhibited by whole blood. We have prepared a virus sterilized FFP (S/D-FFP) by treatment of FFP with 1% tri(n-butyl)phosphate (TNBP) and 1% Triton X-100 at 30°C for 4 hours. Added reagents are removed by extraction with soybean oil and chromatography on insolubilized C18 resin. Treatment results in the rapid and complete inactivation of $\geq 10^{7.5}$ infectious doses (ID_{50}) of vesicular stomatitis virus (VSV) and $\geq 10^{6.9}$ ID_{50} of sindbis virus (used as marker viruses), $\geq 10^{6.2}$ ID_{50} of human immunodeficiency virus (HIV), $\geq 10^{6}$ chimp infectious doses (CID_{50}) of hepatitis B virus (HBV), and $\geq 10^{5}$ CID_{50} of hepatitis C virus (HCV). Immunization of rabbits with S/D-FFP and subsequent adsorption of elicited antibodies with untreated FFP confirmed the absence of neoimmungen formation. Coagulation factor content was comparable with that found in FFP. Based on these laboratory and animal studies, together with the extensive history of the successful use of S/D-treated coagulation factor concentrates, we conclude that replacement of FFP with S/D-FFP, prepared in a manufacturing facility, will result in improved virus safety and product uniformity with no loss of efficacy.

THE RISK OF transmission of hepatitis B virus (HBV), non-A, non-B hepatitis virus (NANBHV; now established as predominantly hepatitis C virus [HCV]), and of human immunodeficiency virus (HIV) by coagulation factor concentrates was greatly diminished with the advent of virus sterilization methods compatible with labile proteins.[1-6] Treatment of coagulation factor concentrates with the organic solvent, tri(n-butyl)phosphate (TNBP), and detergent (sodium cholate, Tween 80, or Triton X-100) was shown to inactivate very large quantities of HBV, HCV, and HIV[7,8] with little or no loss in coagulation factor activity.[8-10] As of February 1991, over 1.7 million doses (1,000 U/dose) of S/D-treated coagulation factor concentrates have been infused (personal communication, Christine Watklevicz, The New York Blood Center, March 1991). Viral safety has been confirmed in at least nine independent clinical trials involving 136 hemophiliacs believed to be susceptible to hepatitis viruses and 245 hemophiliacs believed to be susceptible to HIV, with administration of over 3 million units of coagulation factor.[6] This safety record indicates that S/D-treated concentrates are far safer than the individual units from which they were derived. Further evidence of the safety of S/D-treated products comes from studies of the properties of HIV immune globulin prepared exclusively from plasma collected from members of the homosexual population previously exposed to HIV. After S/D treatment, both plasma and purified immune globulin (HIV-IG) derived exclusively from HIV antibody-positive donors were safe from the transmission of HBV, HCV, and HIV on injection into chimpanzees.[11]

Currently, the viral safety of fresh frozen plasma (FFP) relies solely on donor selection and donor blood screening methods. Experience with coagulation factor concentrates suggests that the safety of FFP can be enhanced on treatment by the solvent/detergent approach, provided a similar level of virus kill can be achieved. This report describes a process for the preparation of S/D-plasma, evidence of HIV, HBV, and HCV kill with this process, and an in vitro assessment of protein integrity.

MATERIALS AND METHODS

Reagents. Chemicals were reagent grade unless otherwise stated. TNBP and Tween 80 were obtained from Fisher Scientific (Springfield, NJ); Triton X-100 was obtained from Serva Feinbiochemica (Heidelberg, Germany); and Prep C18 resin was obtained from the Waters Division of Millipore Corp (Milford, MA).

Virus-inactivation procedure and virus assays. Studies of the inactivation of vesicular stomatitis virus (VSV) and Sindbis virus were performed as described previously.[9] Both viruses are lipid enveloped. Previous studies have shown that solvent/detergent mixtures do not inactivate protein-enveloped virus. The reactions were stopped by 100-fold dilutions of virus into medium (Minimal Essential Medium Eagle; GIBCO Laboratories, Grand Island, NY) containing 5% fetal calf serum. After sterile filtration and storage at −70°C, virus titer was determined by endpoint dilution and cellular cytopathology, using human A549 cells with VSV and primary chicken embryo cells with Sindbis virus. HIV-III_b strain was used to assess the inactivation of HIV added to plasma, as described previously.[12] The reaction was stopped by adding 0.12 g of Waters Prep C18 resin to 1 mL of the treated plasma, mixing for 3 minutes, removing the resin by centrifugation, and repeating the process once. HIV infectivity was assayed by endpoint dilution, measuring reverse transcriptase after cultivation of virus exposed CEM cells for 14, 21, and 28 days.

The inactivation of HBV (strain NYBC 75-564) and NANBHV (strain 59, a passage from the Hutchinson inoculum) on treatment of plasma was studied as described.[7] Plasma from one carefully selected donor who was free of hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), and hepatitis B core antibody (HBcAb), who had an alanine aminotransferase (ALT) level ≤25 IU/L, and who came from a group with a low risk of exposure to hepatitis viruses (ie, middle class, white, and

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heterosexual) was contaminated with either HBV to an end concentration of 10^5 chimpanzee-infectious doses (CID_{50}) per milliliter or NANBHV to an end concentration of 10^4 CID_{50} per milliliter. The virus-spiked plasma solutions were incubated in the presence of 1% TNBP and 1% Triton X-100 in a shaking water bath at 30°C for 4 hours. After 1 hour of TNBP treatment, the plasma-virus mixture was transferred to a second tube to ensure that every droplet of plasma was contacted by the TNBP. The chimpanzees were immediately inoculated without further processing of the samples.

Four chimpanzees were injected with treated plasma: two received plasma spiked with 10^6 CID_{50} of HBV and two received 10^5 CID_{50} of NANBHV. Chimpanzees used for the HBV inactivation study had no previous exposure to blood products and were free of hepatitis B markers. Chimpanzees for the NANBHV inactivation study had been used previously to assess the safety of HBV vaccine. After 9 months, the chimpanzees who received treated HBV-containing plasma were injected with 1 mL of a dilution of the untreated inoculum containing $10^{3.8}$ CID_{50} of HBV. After 6 months, the chimpanzees injected with treated NANBHV-containing plasma received 1 mL of the untreated inoculum containing $10^{3.9}$ CID_{50} of NANBHV. All chimpanzees were observed for an additional 6 months. Weekly sera were assayed for ALT, aspartate aminotransferase, HBsAg, anti-HBs, and anti-HBc. Bimonthly Menghini needle liver biopsies were examined by both light and electron microscopy.

Preparation of S/D-plasma. FFP was thawed rapidly and treated with stirring for 4 hours with 1% (vol/vol) TNBP and 1% (vol/vol) Triton X-100 at 30°C. After treatment, soybean oil (5% vol/vol) was added, mixed gently for 30 minutes, and then removed by centrifugation at 10,000*g* for 20 minutes. The clarified plasma was then applied to a column of Waters Prep C18 resin such that the ratio of plasma to column volume was 6 and the contact time was 3 minutes. The column eluate was filtered on a 0.2-μm filter.

Neoimmunogenicity. New Zealand white rabbits were immunized with S/D-plasma. Each animal was injected subcutaneously on three occasions at 2-week intervals; the first inoculum was emulsified with Freund's complete adjuvant and the subsequent ones with Freund's incomplete adjuvant. To determine whether S/D-plasma elicited antibody nonreactive with untreated plasma, antisera from each animal were collected immediately preceding each injection (bleeds 1 to 3) and five additional times over the subsequent 3 months. Antisera were analyzed by the Ouchterlony technique and by neutralizing crossed immunoelectrophoresis. In the latter case, anti–S/D-plasma was incubated with plasma in a test tube. After removal of the precipitate, remaining soluble antibody was incorporated into a gel to be used as the second dimension of a crossed immunoelectrophoresis. As a control, chicken albumin was added to the S/D-plasma used as the sample for electrophoresis and antichicken albumin was added at the outset of the experiment to the anti–S/D-plasma samples.

Assays. Coagulation factor activity for FVIII, FIX, and FXI was determined by one-stage activated partial thromboplastin (APTT) time clotting assay, which measures the degree of correction of the clotting time of factor-deficient plasma (George King Biomedical, Overland Park, KS) in the presence of APTT reagent (Organon-Teknika, Durham, NC). FV activity was assessed similarly, except thromboplastin with calcium (Sigma Diagnostics, St Louis, MO) replaced the APTT reagent.

TNBP was quantitated after hexane extraction by gas chromatography using a 0.25-in by 2 mm ID by 4-ft glass column packed with 10% SP-1000 on an 80/100 mesh Supelcoport (Supelco, Bellefonte, PA). Triton X-100 was assayed by first extracting it from plasma using a Prep C18 column, eluting with 75% isopropanol, concentrating the eluate with a Speed Vac concentrator (Savant, Farmingdale, NY), diluting the concentrate fivefold with water, and subjecting this sample to high performance liquid chromatography (HPLC) on a 3.9 × 300 mm Bondapak C18 column (Waters) with a Hewlett Packard HP1090 HPLC apparatus (Palo Alto, CA) and the UV detector set at 230 nm. The column was eluted with a water/isopropanol linear gradient.

RESULTS

Marker virus inactivation. The inactivation of all detectable VSV and Sindbis virus added to plasma on treatment with 1% TNBP and 1% Triton X-100 at 30°C was very rapid, occurring within 15 minutes (Fig 1). Total demonstrated inactivation was $\geq 10^{7.5}$ tissue culture infectious doses ($TCID_{50}$) for VSV and $\geq 10^{6.9}$ $TCID_{50}$ for Sindbis virus. Use of 0.3% TNBP or a reaction temperature of 24°C resulted in a substantially slower rate of inactivation of

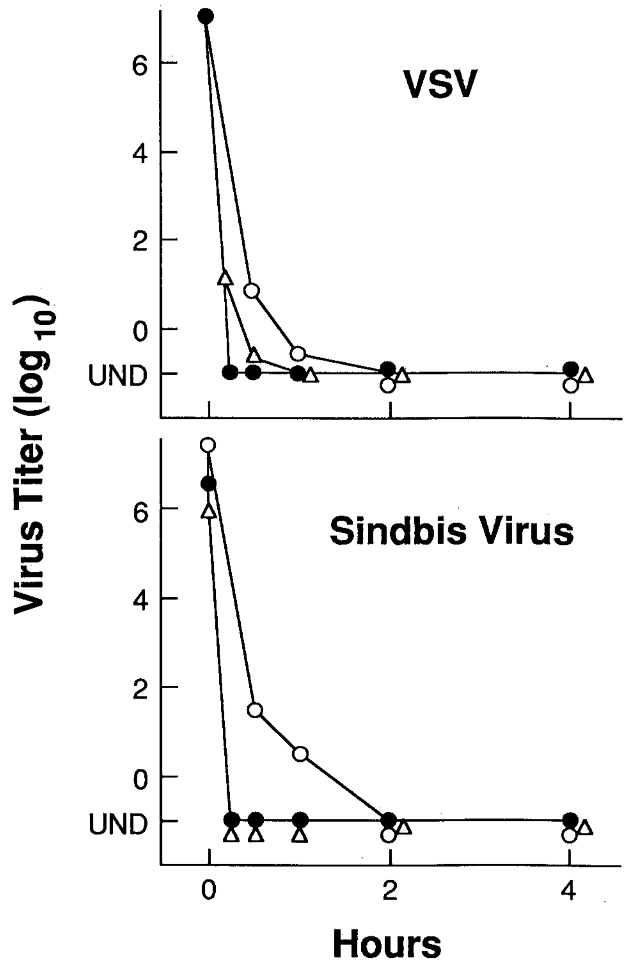

Fig 1. Rate of inactivation of marker viruses added to plasma on treatment with TNBP and Triton X-100. VSV (upper panel) and Sindbis virus (lower panel) were added to human blood plasma (solid circles) or to an AHF concentrate prepared at the New York Blood Center and sampled at the stage in manufacture at which TNBP and detergent are normally added (open circles and triangles). Plasma was treated with 1% TNBP and 1% Triton X-100 at 30°C (solid circles), and AHF was treated with 0.3% TNBP together with either 0.2% sodium cholate at 30°C (open circles) or 1% Tween 80 at 24°C (open triangles). The titer of infectious virus was determined at the indicated times after a 100-fold dilution, used to stop the reaction.

VSV (data not shown). Inactivation of each of these marker viruses on treatment of plasma under our selected conditions was faster than that observed on treatment of an antihemophilic factor (AHF) concentrate with 0.3% TNBP and 0.2% sodium cholate at 30°C or 0.3% TNBP and 1% Tween 80 and 24°C, the conditions in common use today for coagulation factor concentrates (Fig 1). Excellent inactivation of VSV occurred even when added to overtly lipemic plasma. In this case, a small quantity ($10^{0.5}$ $TCID_{50}$) of residual VSV and Sindbis virus was detected after 15 but not after 30 minutes of treatment (data not shown).

HIV. HIV, $\geq 10^{6.2}$ $TCID_{50}$, was inactivated on treatment of plasma with 1% TNBP and 1% Triton X-100 at 30°C for 4 hours (Table 1). A separate experiment showed that HIV inactivation was complete to the limit of detection on exposure to the S/D reagents at the earliest timepoint taken, 1 hour. Additionally, HIV (10^{1} $TCID_{50}$) was removed from plasma on exposure to Waters C18 Prep gel, the resin used in the removal of both TNBP and Triton X-100 during routine processing of S/D-plasma. Thus, the combined efficacy was $\geq 10^{7.2}$ $TCID_{50}$.

HBV. HBV (10^{5} CID_{50}/mL) added to plasma was treated with 1% TNBP and 1% Triton X-100 at 30°C for 4 hours, after which 10 mL was injected immediately into each of two animals. Neither animal developed signs of hepatitis through the initial period of follow-up: 28 weeks for chimpanzee 326, which died of an anesthesia-related incident, and 36 weeks for chimpanzee 327 (Fig 2). The low level of anti-HBs reactivity observed is not considered significant. Following challenge of chimpanzee 327 with the untreated inoculum, $10^{3.8}$ CID_{50} of HBV, a sharp increase in serum ALT level and the appearance of anti-HBs were observed.

HCV. HCV (10^{4} CID_{50}/mL) added to plasma was treated in the manner described for HBV, after which 10 mL was immediately injected into two chimpanzees each. The two animals receiving HCV spiked plasma were monitored for indications of hepatitis transmission for 32 weeks. Throughout this period, serum ALT levels remained below 2 times the upper limit of normal, antibody to the C-100 protein of HCV was undetectable, and liver histology (light and electron microscopy) was normal (Fig 3). To confirm the susceptibility of the animals to HCV, an untreated sample

Table 1. Inactivation of HIV Added to Plasma

Sample	HIV Titer (log_{10} $TCID_{50}$)	HIV Kill or Removal (log_{10} $TCID_{50}$)
Untreated control	7.2	—
4-h incubation, S/D omitted, C18 adsorbed	6.2	1.0
4-h incubation, S/D treated, C18 adsorbed	≤0	≥7.2

HIV, added to plasma, was either mock treated (30°C for 4 hours; top line), mock treated and adsorbed with C18 resin (second line), or treated with 1% TNBP and 1% Triton X-100 at 30°C for 4 hours, after which it was adsorbed with C18 resin (third line). Undiluted and 10-fold serial dilutions of each sample were assayed for infectious HIV as described in Materials and Methods.

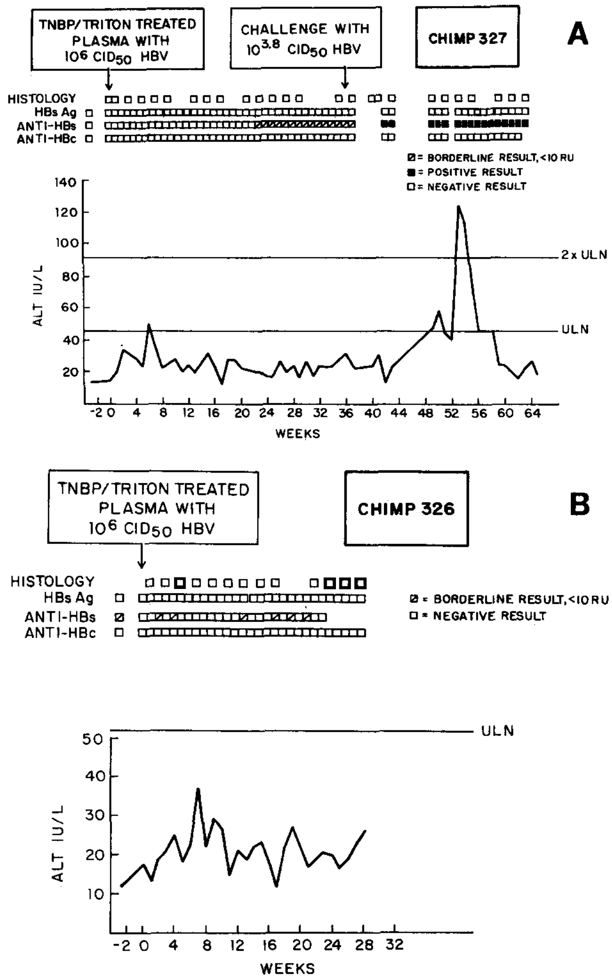

Fig 2. Course of two chimpanzees inoculated with 10^{6} CID_{50} of HBV in TNBP/Triton-treated plasma and then challenged with untreated inoculum containing $10^{3.8}$ CID_{50}. Samples taken at the indicated times were analyzed for markers of hepatitis; liver histology, HBsAg, anti-HBs, anti-HBc, and ALT. Open boxes indicate negative results, boxes with a diagonal line a borderline result, and solid boxes a positive result. ULN denotes the upper limit of normal in ALT values for that animal, as determined before the start of the experiment on 16 separate serum samples.

was injected, using an HCV dose of $10^{3.9}$ CID_{50}/animal. Both animals developed signs of HCV infection, indicating susceptibility. Chimpanzee 298 had evidence of a transient, though sharp increase in serum ALT level, and Pfeiffer structures types I to IV were observed on examination of liver biopsies by electron microscopy. Anti-HCV (C100) was not observed. Chimpanzee 299 exhibited Pfeiffer structures 4 to 14 weeks after challenge and subsequently developed detectable anti-HCV (C100).

Coagulation factor activity. All coagulation factors measured remained at or near the level observed in the control, start sample when FFP plasma was thawed and processed at the 15- to 20-L scale (Table 2). In processing four separate plasma pools, comparison of the start and end concentrations of coagulation factors V, VIII, IX, and XI

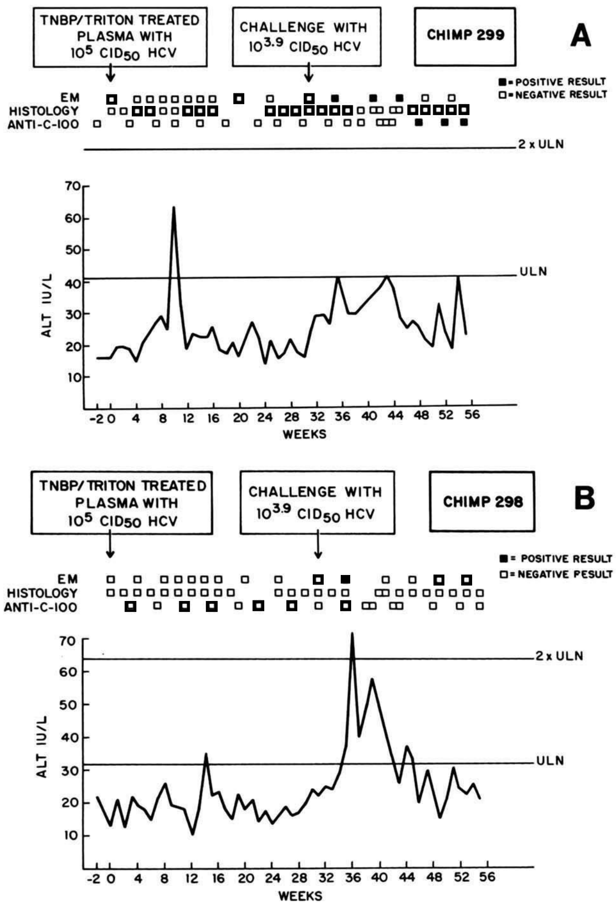

Fig 3. Course of two chimpanzees inoculated with 10^5 CID_{50} of HCV in TNBP/Triton-treated plasma and then challenged with untreated inoculum containing $10^{3.9}$ CID_{50}. Samples taken at the indicated times were analyzed for markers of hepatitis; liver histology by light and electron microscopy, anti-HCV (C-100), and ALT. Other details as in Fig 2.

showed a recovery of 87%, 88%, 99%, and 108%, respectively. Prothrombin times and APTT values were normal, varying in four consecutive lots from 10.5 to 12.1 seconds and 32.0 to 33.8 seconds, respectively. These results suggest that contact factor activation is not encountered during processing. Moreover, factor XII levels were normal (0.78 to 0.88 U/mL; n = 3) in S/D-plasma prepared by Octapharma (Vienna, Austria; personal communication H. Schwinn, September 1991). At the 20-L scale, processing resulted in about a 20% loss in plasma volume.

Absence of neoimmunogenicity. S/D-plasma injected into each of three rabbits did not elicit an antibody reactive with S/D-plasma that did not react with untreated plasma, as judged by the Ouchterlony technique (Fig 4) or by neutralizing crossed immunoelectrophoresis (Fig 5).

Removal of TNBP and Triton X-100 by C18 chromatography. S/D-treated plasma was extracted with 5% soybean oil, subjected to centrifugation at 10,000*g* for 10 minutes to remove lipid, and then applied to a column (14 × 14 cm) of Waters C18 Prep resin. With a contact time of 3 minutes, ≤2 μg/mL of either TNBP or Triton X-100 was present in the eluate when as much as 6 column volumes of plasma

Table 2. Recovery of Selected Coagulation Factors

	Start (U/mL)		End (U/mL)		Recovery
Factor	Average	SD	Average	SD	(%)
Factor V	0.83	0.07	0.72	0.07	87
Factor VIII	0.93	0.06	0.82	0.04	88
Factor IX	0.98	0.07	0.97	0.05	99
Factor XI	0.86	0.05	0.93	0.10	108

FFP (15 to 20 L) as thawed, treated with 1% TNBP and 1% Triton X-100 at 30°C for at least 4 hours, and passed through a column of Waters C18 Prep Gel as described in Materials and Methods. Coagulation factor activities of the freshly thawed plasma and the C18 eluate were measured and compared (n = 4; SD = standard deviation).

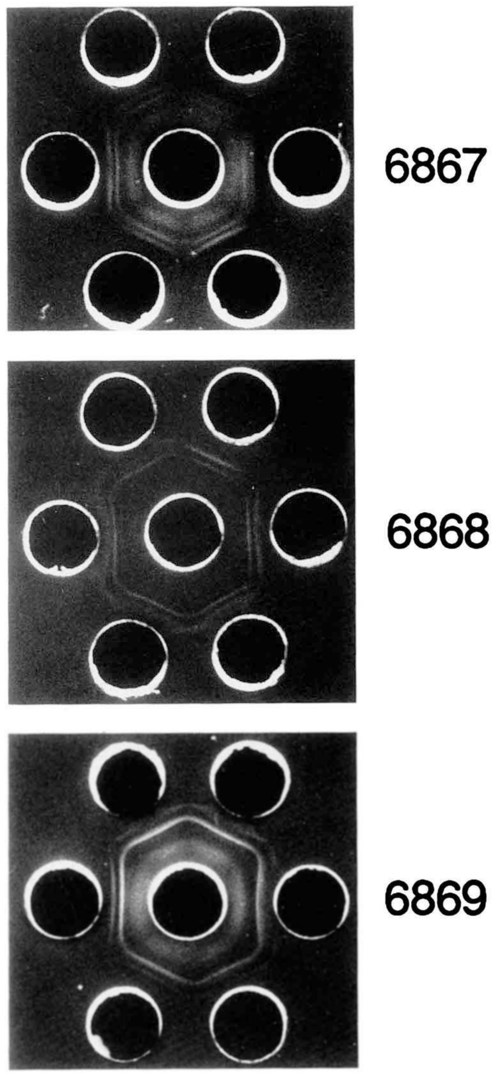

Fig 4. Ouchterlony analysis of S/D-plasma. Rabbit antibodies raised against S/D-plasma (center wells) were allowed to react with serial dilutions of untreated plasma and S/D-plasma alternating in wells at the circumference. Sera from the fifth bleed from each of the three immunized rabbits (numbers 6867, 6868, and 6869) were used.

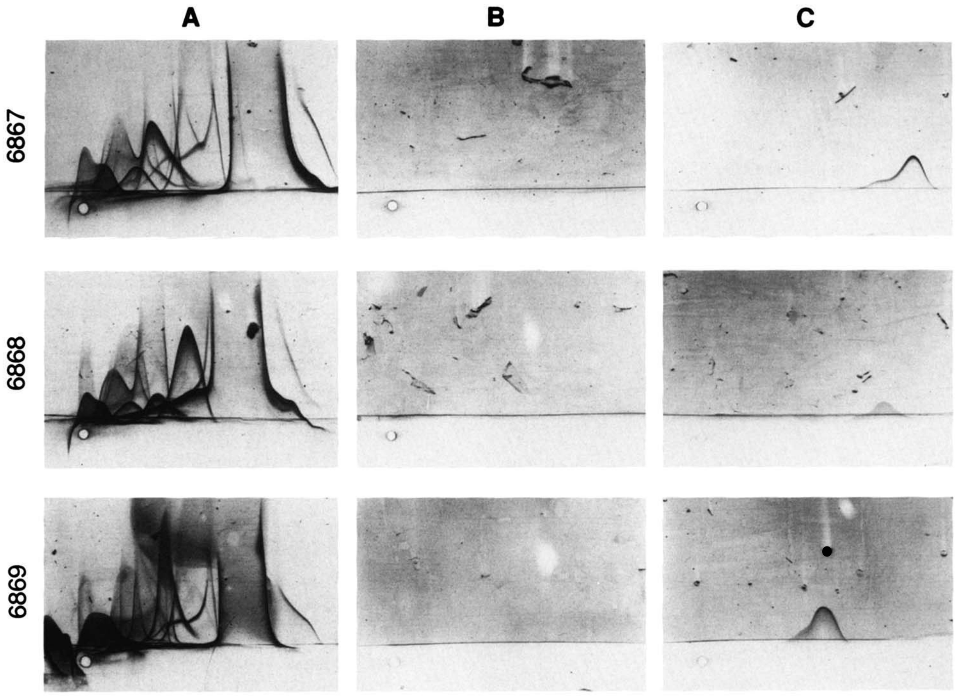

Fig 5. Neutralizing crossed-immunoelectrophoresis of S/D-plasma. S/D-plasma, as antigen, was electrophoresed in the first dimension, except for the right panels, where chicken albumin was also included. Antibody incorporated into the gel used in the second dimension of the electrophoresis was anti-S/D-plasma (left panels), anti-S/D-plasma preadsorbed with untreated plasma (middle panels), and anti-S/D-plasma preadsorbed with untreated plasma plus antichicken albumin (right panels). Sera from the fifth bleed of each of the three immunized rabbits (numbers 6868, 6868, and 6869) were used.

treated with 1% TNBP and 1% Triton X-100 were applied (data not shown).

DISCUSSION

Approximately 2 million units of FFP are used in the United States each year. While it is generally conceded that much of this usage is inappropriate, a Consensus Conference sponsored by the National Heart Lung Blood Institute concluded that FFP is useful in the treatment of coagulation factor disorders where appropriate concentrates are unavailable and when multiple coagulation factor deficits occur, such as in surgery.[13] Since that meeting, FFP usage in the United States has remained essentially constant. If FFP is to be transfused, it should be made as safe as possible even while efforts continue to eliminate inappropriate usage.

We have now shown that treatment of pooled plasma with 1% TNBP and 1% Triton X-100 for 4 hours at 30°C inactivates $\geq 10^6$ CID_{50} of HBV, $\geq 10^5$ CID_{50} of HCV, and $\geq 10^{6.2}$ $TCID_{50}$ of HIV. Subsequent exposure to the C18 column used to remove TNBP and Triton X-100 was shown to remove an additional 10^1 $TCID_{50}$ of HIV. Furthermore, because the rate of inactivation of VSV is faster in this system than observed on treatment of an AHF concentrate with either 0.3% TNBP and 0.2% sodium cholate at 30°C or 0.3% TNBP and 1% Tween 80 at 24°C (conditions in use worldwide), it seems likely that S/D-plasma will enjoy the same record of virus safety.

While considerable data support the effectiveness of the S/D approach against lipid-enveloped viruses, nonenveloped viruses, should they be present, would not be inactivated. The example most frequently raised in this context is parvovirus B19. Fortunately, B19 infections are self-limiting and largely asymptomatic.[14,15] While not considered to be a serious threat in most transfusion settings, antiparvovirus antibody expected to be present in more than half of the units[15] might effectively inactivate the rare unit[16] containing parvovirus, depending on the respective titers of antibody and antigen. This conclusion seems plausible because routinely prepared IVIG preparations have been shown to neutralize parvovirus in vitro and in vivo.[17]

The protein content of S/D-plasma prepared from large pools of FFP appears to be normal in all respects. Initial preparation at the 20-L scale resulted in excellent maintenance of the levels of coagulation factors V, VIII, IX, and XI, and of PT and APTT values. Neoimmunogens were absent when analyzed in a rabbit model. Additionally, the considerable worldwide experience with S/D-treated products indicates that the proteins present in S/D-plasma will circulate and function normally in vivo.

Finally, it should be noted that TNBP and Triton X-100 were efficiently removed by the described process. Residual values were ≤ 2 μg/mL of each when processing at the 20-L scale, and always ≤ 10 μg/mL when processing was performed at larger scale. If present at 10 μg/mL, a 70-kg man receiving 2 L of S/D-plasma could potentially be exposed to 0.29 mg/kg body weight. This is more than 2 orders of magnitude lower than the reported lowest effect dose on intraperitoneal injection of TNBP into mice[18] and intravenous injection of Triton X-100 into mice.[19] Results on the acute toxicity of TNBP and Triton X-100 in mice and rats from studies jointly sponsored by the New York Blood Center and Octapharma (Dusseldorf, Germany) conducted by the Laboratory of Pharmocology and Toxicology (Hamburg, Germany) are in accord with these earlier, published studies (unpublished results, October 1991). Soybean oil is used as a principal ingredient in lipid emulsions designed for total parenteral nutrition. It consists almost entirely of triglycerides naturally occurring in humans, largely those of linoleic, oleic, and linolenic acid, and daily intravenous administration of more than 2 g/kg body weight of lipid emulsion has proven to be safe.[20,21] By contrast, we have found no more triglyceride in the treated solution than that normally found in plasma. Toxicity should not be associated with the use of the Prep C18 resin. It is prepared by reaction between octadecyldimethyl silanol and the silica backbone. Information from the manufacturer (Millipore Corp, Waters Chromatography Division) indicates that leakage of this functional group should be very low, and none was found in S/D-plasma when analyzed by Waters with an assay whose sensitivity was 1 μg/mL.

Based on these findings, lots for use in clinical studies are now being prepared by the Melville Biologics Division of the New York Blood Center at the 60- to 250-L scale. Because of the nature of the process, preparation is best performed in a traditional manufacturing rather than a blood bank environment. Preliminary results from clinical studies indicate the absence of side effects of this new preparation (M.S. Horowitz, personal communication, October 1991).

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