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STERILISATION OF HEPATITIS AND HTLV-III VIRUSES BY EXPOSURE TO TRI(n-BUTYL)PHOSPHATE AND SODIUM CHOLATE

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Summary Blood product sterilisation with 0·3% tri(n-butyl)phosphate (TNBP)/0·2% sodium cholate (CA), a combination known to permit high recovery of factor VIII and factor IX, was evaluated for its effect on hepatitis B (HBV), non-A, non-B (NANB), and human T-lymphotropic type III (HTLV-III) viruses. 2 chimpanzees received factor VIII preparations contaminated with 10^4 chimpanzee infectious doses (CID_{50}) of HBV and treated with TNBP/CA; neither had evidence of HBV infection during 9 months follow-up, but hepatitis B surface antigen (HBsAg) developed 5 and 6 weeks, respectively, after challenge with untreated inoculum. 2 chimpanzees were similarly exposed to 10^4 CID_{50} of Hutchinson NANB inoculum treated with TNBP/CA; neither became infected during 26 weeks of follow-up but both had characteristic NANB-associated ultrastructural changes 3–5 weeks after exposure to untreated inoculum. 2 chimpanzees inoculated with 80 ml of TNBP/CA-treated factor VIII derived from a pool of thirteen lots obtained from five US manufacturers remained free of any evidence of NANB infection during 32 weeks of follow-up. Subsequently, NANB infection developed in both animals 3–4 weeks after exposure to untreated inoculum. Exposure of HTLV-III diluted into a factor VIII preparation to TNBP/CA inactivated $>10^{4\cdot2}$ tissue culture infective doses within 20 min at 24°C.

Introduction

RECOGNITION that AIDS could be transmitted by blood and blood products[1–3] provided a long overdue urgency to implementation of safe and effective means for sterilisation of blood derivatives. It became essential that preparation of high-risk blood derivatives should include procedures to inactivate human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV)[4–7] as well as the previously identified hazards of hepatitis B (HBV), and

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non-A, non-B (NANB) hepatitis viruses. Moreover, satisfactory sterilisation procedures should be free of toxicity, and have little or no detrimental effect on the proteins treated. Lack of protein denaturation is important for two reasons: particularly for factor VIII, reductions in yield increase cost and decrease availability; and denatured proteins may expose cryptic neoantigens which would give rise to inhibitory antibodies or hypersensitivity.

Various approaches have been adopted to meet these needs. In Germany, Biotest has for many years used the combination of β-propiolactone and ultraviolet irradiation for sterilisation of plasma.[8,9] This process is highly effective for inactivation of HBV and NANB,[10–12] but because of difficulties with yields, has not yet been applied commercially to production of factor VIII. In the USA, most manufacturers now pasteurise products in the dry state, but this approach has little effect on inactivation of HBV[13] and products which have been heated in the dry state have been shown to transmit NANB to patients.[14,15] However a product treated in this way did not appear to transmit HTLV-III.[16] Behringwerke in Germany has used a pasteurisation process in which factor VIII is heated for 10 h at 60°C in the liquid state in the presence of glycine and sucrose as stabilisers;[17] the efficacy of this procedure with respect to inactivation of NANB virus(es), or yields of factor VIII is unknown.

We have studied inactivation of HBV, NANB, and HTLV-III in blood by sterilisation with the organic solvent tri(n-butyl)phosphate (TNBP) and the detergent sodium cholate (CA). The procedure was devised as a result of our previous finding that a solvent/detergent combination ('Tween' 80/ether) was exceptionally effective for inactivation of hepatitis viruses.[18] The tween/ether process, however, has the disadvantages that ether is inflammable while tween 80 is difficult to remove from factor VIII preparations. The new procedure was adopted after extensive preliminary studies with easily quantifiable marker viruses.[19] Biochemical studies had documented high recovery of factor VIII and factor IX in the absence of any protein denaturation, and the treating agents could be readily removed from the treated product.[19]

Materials and Methods

Preparation of New York Blood Center Factor VIII Pool

Factor VIII concentrate was prepared from 4500 ml of freshly frozen plasma obtained from eight carefully selected donors who were free of HBsAg, hepatitis B surface antibody (HBsAb), and

hepatitis B core antibody (HBcAb); had alanine aminotransferase (ALT) levels <20 IU/l; and came from groups with a low risk of exposure to hepatitis viruses (middle-class, Caucasian, heterosexual).

Anti-haemophilic factor (AHF) was prepared as previously described.[20] Briefly, plasma units were thawed overnight at 4°C and cryoprecipitates were isolated by centrifugation in the cold. Pooled cryoprecipitate was washed at 0°C with 0·02 mol/l 'Tris'-HCl, pH 7·0, and then dissolved in the same solution at room temperature and adsorbed with $Al(OH)_3$. After adjustment to pH 6·5, the solution was brought to 10°C for 15 min and the precipitate removed by centrifugation at 10°C. The supernatant was then adjusted to 0·05 mol/l NaCl and pH 7·0; clarified by centrifugation; adjusted to 110 ml with 0·02 mol/l tris, 0·02 mol/l citrate, 0·05 mol/l NaCl, pH 7·0; sterile filtered; and frozen in aliquots at −80°C.

AHF content was 5 U/ml, and the absorbance at 280 nm was 6·6.

Addition of HBV and Treatment with TNBP/CA

30 ml of AHF concentrate was thawed, clarified, and contaminated with 2000 CID_{50}/ml of HBV by addition of 191 µl of rapidly thawed HBV/ay (MS-2 strain) 99–8 inoculum (National Institutes of Health, Bethesda, Maryland), by use of a 10^{-2} dilution of the original stock which contained $10^{7\cdot5}$ CID_{50}/ml.[21] Two 2·5 ml aliquots were immediately quick frozen and held at −80°C for subsequent use as challenge inocula. 22 ml of virus-containing AHF was then treated with 0·92 ml 5% CA and 66 µl of TNBP to bring the concentration to 0·2% (w/v) CA and 0·3% (v/v) TNBP, and held at 30°C for 6 h with stirring. CA and TNBP were removed by passing the solution through a 1·6×96 cm 'Sephadex' G-25 column, equilibrated with 0·02 mol/l tris-HCl, pH 7·2, 0·02 mol/l sodium citrate, 0·1 mol/l NaCl at a flow rate of 20 ml/cm²/h. The void volume containing AHF was pooled, adjusted to 40 ml, sterile filtered, and rapidly frozen at −80°C.

Addition of Hutchinson Strain NANB and Treatment with TNBP/CA

Hutchinson NANB inoculum[22] was rapidly thawed by swirling in a 37°C waterbath. 1 ml of this inoculum infected 2/2 chimpanzees at a 10^{-6} dilution,[22] and 0/2 at a 10^{-7} dilution (B. Brotman, A. M. Prince, unpublished), therefore this inoculum has a titre of $10^{6\cdot5}$ CID_{50}/ml.

30 ml of the AHF concentrate described above was contaminated by addition of 18·8 µl to bring the virus content to 2000 CID_{50}/ml. After removal of aliquots to be used for challenge, the remaining solution was treated with TNBP/CA, and passed through a sephadex G-25 column as described above.

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Preparation of Pool of Commercial Factor VIII Preparations and Treatment with TNBP/CA

Thirteen lots of factor VIII concentrate from five US manufacturers were pooled aseptically and adjusted from 340 ml to 500 ml by addition of 160 ml of 0·02 mol/l tris-HCl pH 7·2, 0·02 mol/l sodium citrate. Aliquots were removed and rapidly frozen for use in subsequent challenge experiments. 6·25 ml of 5% (w/v) CA and 0·45 ml of TNBP were added to 150 ml of the pool, which was then held at room temperature for 6 h with stirring. TNBP and CA were removed by passage through a 5×89 cm sephadex G-25 column, equilibrated with 0·02 mol/l tris-HCl pH 7·2, 0·02 mol/l sodium citrate, 0·1 mol/l NaCl at a flow rate of 20 ml/cm²/h. The void volume was recovered, adjusted to twice the volume of the applied sample, sterile filtered, and quick frozen at −80°C.

Chimpanzees

6 wild-caught juvenile chimpanzees aged 3–5 years held in the New York Blood Center's primate facility (Vilab II) at the Liberian Institute for Biomedical Research, Robertsfield, Liberia, were the subjects of the present trial. The animals had been held in quarantine for 2–4 years before the study began and were free of HBsAg, HBsAb, and HBcAb, as detected by biweekly blood specimens. Animals were housed in pairs in large, screened outdoor cages that permitted freedom of movement and play. Extensive precautions were taken to prevent transmission of infections from humans to animals and vice versa, as well as from cage to cage. Precautions included wearing of disposable or sterilisable gloves, boots, and coveralls; sterilisation of all food containers after each use; use of cleaning brushes in one cage only; use of disposable covers on tables used for bleeding and biopsies; use of disposable bleeding and anaesthetic supplies, &c.

The 2 animals used in the HBV experiment had not been used in any previous study. The 4 animals used in the NANB experiments had been used in hepatitis B vaccine safety tests.

Follow-up of Inoculated Animals

Each animal was bled weekly except in NANB studies for which animals were bled twice weekly for the first 6 weeks. Ketamine hydrochloride anaesthesia was used when required. 10 ml of blood were collected and allowed to clot, and serum was promptly separated. Liver biopsies (Menghini needle) were obtained weekly for the first 6 weeks in NANB studies and on all animals at 2-week intervals. Formalin-fixed biopsies were embedded in paraffin and stained with haematoxylin and eosin.

A portion of each biopsy was fixed with buffered glutaraldehyde and OsO_4 and embedded in 'Epon' 812 (Shell Chemical, Houston, Texas). Monthly biopsies from animals in NANB experiments were sectioned and examined in a Phillips model 410 electron microscope for characteristic ultrastructural changes of NANB infection in chimpanzees.[23]

Diagnosis of Hepatitis

Aminotransferase determinations.—Serum was tested on the day of collection at Vilab II for ALT and aspartate aminotransferase (AST) by kinetic spectrophotometric assay with a 'Stazar' III kinetic analyser (Gilford Instrument, Oberlin, Ohio) with reagents and reference standards from Worthington Biochemical (Freehold, New Jersey). Normal values for these assays as determined by tests on 95 normal chimpanzees in our colony were: for ALT, geometric mean = 14·8 IU/l, normal range (antilog mean log ALT±2 SD) = 8·9–24·6; for AST, geometric mean = 12·3 IU/l, normal range = 5·6–26·9. All abnormal results were repeated, and results were averaged.

After testing, remaining serum was frozen at −70°C and shipped on dry ice to New York where additional ALT determinations were done as above with an Abbott ABA 200 enzyme analyser (Abbott Laboratories, North Chicago, Illinois) with Abbott reagents and Beckman standards.

Aminotransferase determinations exceeding the upper limit of normal for each animal (based on mean±3 SD of at least twelve consecutive tests carried out before entry into protocol) on two successive occasions, with values for ALT greater than those for AST, were considered diagnostic for hepatitis if accompanied by the histological features of acute hepatitis.

Serological methods.—Sera collected weekly were tested for HBsAg by ELISA (Auszyme; Abbott Laboratories) at Vilab II and for HBsAb (Ausab; Abbott Laboratories) and HBsAg (Ausria-II) at New York Blood Center. Monthly sera, and sera collected weekly around the time of seroconversion, were tested for HBcAb by a solid-phase competitive-inhibition radioimmunoassay (Corab; Abbott Laboratories).

Assay for HTLV-III

Infectivity of HTLV-III was assayed quantitatively by inoculation of serial dilutions into H-9 cell-cultures as previously described,[24] except that anti-α-interferon was not used in the culture medium. To enhance the sensitivity of the assay, undiluted samples (5 ml) were also inoculated into 40 ml cultures. Whereas the endpoint in titrations done in 0·2 or 2·0 ml cultures is reached in 14 days,[24] a 3–4 week follow-up is required to reach titration endpoints with the larger cultures. Sensitivity of large and small cultures is the same when 4 weeks of follow-up are used (unpublished observations).

Results

Inactivation of HBV

When 10^4 CID_{50} HBV suspended in factor VIII concentrate was treated with TNBP/CA for 6 h and inoculated intravenously into 2 chimpanzees, neither showed

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evidence of hepatitis or HBV infection during 40 weeks' of follow-up (fig 1). When the animals were challenged with 1·0 ml of untreated HBV-contaminated AHF concentrate containing an estimated 2000 CID_{50}, typical HBV infections developed in both, with incubation periods before appearance of HBsAg of 5 and 6 weeks, respectively. These incubation periods are within the expected range (6·7±1·3 weeks) for inocula containing $10^{3·5}$ CID_{50} of HBV strain,[10] and confirm the titre of the inoculum.

Inactivation of Hutchinson Strain NANB

Of 2 chimpanzees inoculated intravenously with factor VIII concentrate containing 10^4 CID_{50} of Hutchinson strain NANB and treated with TNBP/CA, neither showed any evidence of hepatitis or NANB infection by electron microscopy over 26 weeks of intensive observation (fig 2). After challenge with intravenous inoculation of 2·5 ml of untreated factor VIII concentrate containing an estimated 2000 CD_{50}/ml, typical NANB hepatitis developed in both animals, with characteristic ultrastructural changes appearing by 3·5 weeks. These findings confirmed the susceptibility of the animals, and the infectivity of the inoculum.

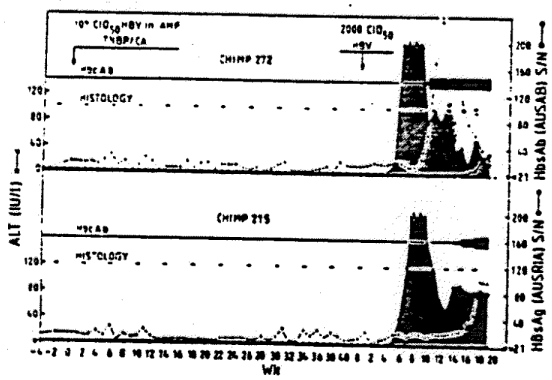

Fig 1—Course of 2 chimpanzees inoculated with 10^4 CID_{50} of HBV in TNBP/CA-treated factor VIII and then challenged with untreated inoculum containing 2000 CID_{50}.

HbcAb is denoted by a thin line when negative and by a thicker horizontal bar when positive; width of the bar is proportional to the degree of positivity. *S/N* (signal/noise) denotes the division of the observed cpm in the sample by the observed background.

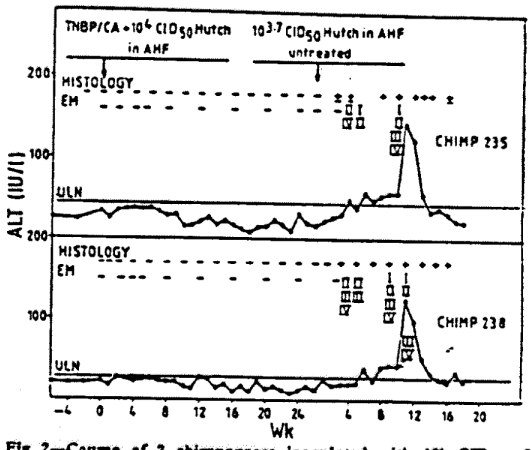

Fig 2—Course of 2 chimpanzees inoculated with 10^4 CID_{50} of Hutchinson strain NANB treated with TNBP/CA and then challenged with untreated inoculum containing 2000 CID_{50}.

Roman numerals refer to ultrastructural changes of Pfeifer et al.[23] ULN=upper limit of normal.

Inactivation of Commercial Factor VIII Pool

TNBP/CA treatment can only inactivate membrane-coated viruses. The report by Bradley and colleagues of a chloroform-resistant, presumably protein-coated, NANB agent isolated from an infected chimpanzee[25] was thus of great interest. To determine whether such an agent exists in human plasma, we prepared a pool of commercial factor VIII preparations as described above. Clinical studies have indicated that most lots of untreated factor VIII concentrate contain NANB viruses.[26,27]

Of 2 animals injected intravenously with 80 ml of the treated pool, both remained free of any evidence of hepatitis or NANB infection throughout 32 weeks of intensive follow-up; no ALT alterations, ultrastructural changes of NANB infection, or histological evidence of hepatitis were observed (fig 3). Both animals were then challenged with 10 ml of untreated pool, and typical NANB hepatitis promptly developed. Chimpanzee 194 had abnormal ALT levels beginning at 3 weeks and persisting at least 10 weeks; these were accompanied by typical tubular type ultrastructural changes and by acute hepatitis seen by light microscopy. In chimpanzee 229, ultrastructural changes appeared at 4

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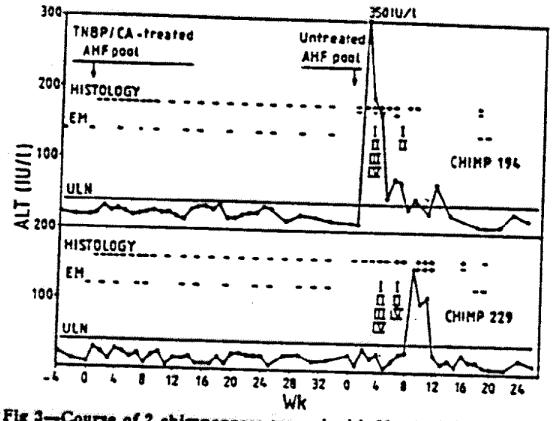

Fig 3—Course of 2 chimpanzees treated with 80 ml of TNBP/CA-treated factor VIII derived from a pool and then challenged with untreated pool.

Roman numerals refer to ultrastructural changes of Pfeifer et al.[23] ULN = upper limit of normal.

weeks, acute hepatitis was seen histologically at 7 weeks, and ALT abnormalities began at 8 weeks.

Inactivation of HTLV-III in Factor VIII Concentrates

We have previously shown that TNBP/CA can inactivate $\geq 10^{4 \cdot 5}$ $TCID_{50}$ of HTLV-III in 2·5 h at 27°C.[24] To study the kinetics of this inactivation and to assess the extent of inactivation at 24°C, we did a similar study in which an HTLV-III stock containing 10^6 $TCID_{50}$/ml was diluted one in ten into an AHF preparation. A portion of the mixture was treated with TNBP/CA and the mixtures were transferred to a 24°C water bath. At intervals aliquots were passed through a 20 ml sephadex G-25 column to remove TNBP/CA and held at 4°C until assayed for HTLV-III infectivity.

As shown in tables I and II, maximum inactivation was achieved by 20 min even at 24°C. This contrasts with several lipid-membrane-coated model viruses used in our preliminary studies which require at least several hours for substantial ($\geq$4 $\log_{10}$) inactivation.[19] Thus, HTLV-III is exceptionally susceptible to inactivation by TNBP/CA.

Discussion

This study shows that exposure of labile blood derivatives to TNBP/CA (a procedure previously noted by us to be

TABLE I—EFFECT OF TNBP/CA ON HTLV-III IN AHF AT 24°C: RESULTS OF 24 WELL-PLATE ASSAYS AT 21 DAYS

Group	Material	Incubation at 24°C (min)	Reverse transcriptase, cpm × 10^3, dilution tested (0·1 ml/inoculum)*								TCID$_{50}$/ml	Log$_{10}$ kill†
			10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
1	HTLV-III stock no 4	:	:	:	256	19	81	8	4	6	$10^{6 \cdot 0}$	:
		:	:	:	263	165	624	991	4	8		
2	HTLV-III 1 in 10 in AHF	:	:	456	512	55	981	4	4	:	$10^{5 \cdot 0}$	:
		:	:	293	274	247	4	4	4	:		
3	HTLV-III in TNBP/CA-treated AHF‡	20	3	4	4	4	6	5	:	:	$<10^1$	$>2 \cdot 5$
			5	4	4	3	4	4	:	:		
4	HTLV-III in TNBP/CA treated AHF‡	60	3	3	3	3	3	3	:	:	$<10^1$	$>2 \cdot 5$
			3	9	3	4	3	3	:	:		
5	HTLV-III in TNBP/CA treated AHF‡	120	5	3	3	:	:	:	:	:	$<10^1$	$>2 \cdot 5$
			7	3	3	:	:	:	:	:		
6	HTLV-III in TNBP/CA treated AHF‡	300	5	4	3	:	:	:	:	:	$<10^1$	$>2 \cdot 5$
			3	3	5	:	:	:	:	:		
7	HTLV-III 1 in 10 in AHF‡	180	:	380	590	4	5	3	3	:	$10^{3 \cdot 5}$	:
			:	344	246	4	5	3	4	:		

*X of 20 negative samples 3·8×3=11·5, therefore $\geq$11·5 considered positive. †Relative to sample no 7. ‡Passed through sephadex G-25.

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TABLE II—EFFECT OF TNBP/CA ON HTLV-III IN FACTOR VIII: RESULTS OF MACROCULTURES. 5·0 ml INOCULUM

Group	Material	Incubation at 24°C (min)	Reverse transcriptase (cmp × 10^3)			Log_{10} kill§
			14 days*	21 days†	28 days‡	
3	HTLV-III in TNBP/CA-treated AHF	20	155 (−)	3 (−)	5 (−)	⩾4·2
4	"	60	197 (−)	3 (−)	6 (−)	⩾4·2
5	"	120	122 (−)	3 (−)	6 (−)	⩾4·2
6	"	360	73 (−)	2 (−)	4 (−)	⩾4·2

*3 × neg mean = 267, ⩾267 considered positive.
†3 × neg mean = 11·5, ⩾11·5 considered positive.
‡3 × neg mean = 11·7, ⩾11·7 considered positive.
§Relative to titre of sample no 7 (table 1), ie, $10^{3\cdot1}$ $TCID_{50}$/ml = $10^{4\cdot2}$/5 mL
(−) indicates culture considered negative for HTLV-III infectivity.

associated with high recovery of coagulation factor activities and little or no protein denaturation[19]) effectively inactivates HBV, NANB, and HTLV-III viruses, although the present experiments provide only minimum estimates of efficacy—⩾10^4 for HBV and NANB viruses and ⩾$10^{4\cdot2}$ for HTLV-III. Additional experiments with more potent inocula are necessary to establish limits of inactivation efficacy against these agents. To provide absolute safety for long-term users, a process efficacy of ⩾5–6 log_{10} is preferable.

The major weakness of the solvent/detergent approach to sterilisation of blood derivatives is inability of the process to inactivate non-lipid-membrane-coated viruses. This would not be a serious drawback, however, if such agents were of no importance in blood-transmitted infections. Bradley and colleagues have reported a chloroform-resistant NANB agent isolated from a chimpanzee inoculated with a human factor VIII preparation.[25] To determine whether we could isolate such an agent, we inoculated chimpanzees with TNBP/CA-treated factor VIII derived from a pool of thirteen lots from five different manufacturers but the treated material did not produce hepatitis in 2 inoculated chimpanzees whose susceptibility was confirmed by subsequent exposure to the untreated pool. Thus, we are unable to confirm the existence of a TNBP/CA-resistant NANB agent in a pool of about 15 000 donations. It is possible that the Bradley agent originated in the chimpanzee from which it was isolated, rather than from the human inoculum; alternatively, the agent is uncommon, or easily neutralised by antibody in large pools.

Human parvoviruses have been transmitted occasionally by transfusion and by pooled factor VIII.[28] These agents are believed to be of minor clinical importance except in patients with chronic anaemia in whom aplastic crises can be induced. Human parvoviruses would not be inactivated by TNBP/CA. This minor disadvantage is, however, offset by the excellent ability of the process to sterilise labile blood derivatives with respect to the major hazards associated with their use—HBV, NANB, and HTLV-III.

TNBP/CA-treated factor VIII has now been licensed by the Food and Drug Administration in the USA. Careful clinical surveillance of newly diagnosed haemophiliacs who receive TNBP/CA-treated factor VIII concentrate will be necessary to confirm the sterility of this product.

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