

## Thermal Inactivation of Human Immunodeficiency Virus in Lyophilised Blood Products Evaluated by ID<sub>50</sub> Titrations

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**Abstract.** Inactivation of human immunodeficiency virus (HIV) in lyophilised small pool cryoprecipitate, factor VIII concentrate, prothrombin complex and C<sub>1</sub>-esterase inhibitor concentrate by prolonged heat treatment (72 h, 60° C) was studied. Plasma products, inoculated prior to lyophilisation, had infectious titres ranging from 10<sup>7</sup> to 10<sup>10.5</sup>. Residual infectivity (TCID<sub>50</sub>) was assessed by multiple titrations on H9 cells in a macro system and subsequent detection of virus replication by determining reverse transcriptase activity. Kinetics of inactivation showed a biphasic pattern: during the first 8 h a variable TCID<sub>50</sub> reduction up to 10<sup>4.3</sup> was observed, followed by an additional loss of 10<sup>1</sup>-10<sup>2.7</sup> during the next 64 h. Heat treatment for 72 h resulted in a mean TCID<sub>50</sub> reduction of 10<sup>5</sup>. It is concluded that prolonged heat treatment may lead to the adequate prevention of HIV transmission by lyophilised plasma products.

### Introduction

Epidemiological data have strongly implicated a novel human retrovirus, formerly designated HTLV-III/LAV and recently renamed human immunodeficiency virus (HIV), as the aetiological infectious agent in AIDS [1, 2]. HIV can be transmitted by sexual contact and by transfusions of blood or plasma products [1-3]. Indeed, patients with haemophilia who are dependent on the administration of large quantities of clotting factor concentrates, prepared from large plasma pools, have been shown to be at high risk for infection with HIV and development of AIDS [4-7]. In order to prevent transmission of HIV by anti-haemophilic factor concentrates, it has been recommended that heat-treated factor VIII concentrate be preferentially used [8]. This recommendation was based on preliminary observations showing that HIV is sensitive to heat inactivation [9]. In this study we inoculated plasma products with high titres of HIV and subsequently evaluated the effect of lyophilisation and heat treatment at 60°C for 8-72 h by infectivity assays (TCID<sub>50</sub>).

### Materials and Methods

#### *Inocula*

HIV (strain HTLV-III<sub>B</sub>) was grown in H9 cells (kindly provided by Dr. R. C. Gallo, Bethesda, Md. USA) that were cultured in Iscove modified Dulbecco's medium supplemented with 10% fetal calf serum and antibiotics. Virus inocula were prepared by concentration of culture supernatants of recently infected H9 cells either by precipitation with 10% polyethylene glycol (PEG) 6000 (Baker Chemicals) or by ultracentrifugation (Kontron TST 28 Rotor, 2 h, 25,000 rpm, 5°C). Pelleted virus was resuspended in a 10-mM Tris buffer, containing 100 mM NaCl and 1 mM EDTA, pH 7.8, and was used fresh.

#### *Inoculation, Lyophilisation and Heat Treatment of Plasma Products*

For the evaluation of HIV inactivation during lyophilisation and dry heat treatment of plasma products, virus inocula and liquid plasma products were mixed in a 1:19 (vol/vol) ratio. The following matrices were used: small pool cryoprecipitate, large pool FVIII concentrate, prothrombin complex and C<sub>1</sub>-esterase inhibitor concentrate. The inoculated preparations were aliquoted in 10-ml portions. Lyophilisation was carried out during 48 h at a shelf temperature of 20°C and a pressure decreasing from 0.3 to 0.03 mbar. Moisture content in simultaneously lyophilised dummy preparations was measured by the Karl Fischer method. Upon lyophilisa-

**Table I.** TCID<sub>50</sub> reduction of HIV by lyophilisation of blood product matrices

Exp. No.	Matrix	TCID <sub>50</sub>	
		pre-lyophilisation	post-lyophilisation
1	small pool cryoprecipitate	ND	10 <sup>7.5</sup>
2	factor VIII concentrate	10 <sup>9.0</sup>	10 <sup>7.8</sup>
3	prothrombin complex	10 <sup>9.5</sup>	10 <sup>6.8</sup>
4	C <sub>1</sub> -esterase inhibitor concentrate	10 <sup>10.5</sup>	10 <sup>7.3</sup>
5	prothrombin complex	10 <sup>7.0</sup>	10 <sup>4.6</sup>
6	prothrombin complex	10 <sup>7.6</sup>	10 <sup>4.9</sup>

ND = not determined.

**Table II.** Thermal inactivation of HIV: cumulative reduction of TCID<sub>50</sub> (-log P<sub>t</sub>/P<sub>0</sub>)<sup>1</sup>

Exp. No.	Virus stock <sup>2</sup>	Matrix	Heat treatment, h				
			8	24	32	48	72
1	PEG	small pool cryoprecipitate	4.0	ND	4.0	ND	5.6
2	PEG	factor VIII concentrate	4.3	4.3	ND	5.3	5.3
3	PEG	prothrombin complex	3.3	3.3	ND	4.8	4.8
4	UC	C <sub>1</sub> -esterase inhibitor concentrate	1.5	2.8	ND	2.8	4.2

ND = not determined.

<sup>1</sup> P<sub>0</sub> = untreated inoculum ID<sub>50</sub>; P<sub>t</sub> = heat-treated ID<sub>50</sub>.<sup>2</sup> Virus concentrated by PEG or ultracentrifuge pelleting.

tion, the vials, sealed under vacuum, were heated in a water bath at 60°C (±0.5°C) for 8–72 h. Temperature was recorded in dummy vials. Under these conditions, similar to production procedures, the characteristics of the lyophilised plasma products did not change significantly, with only a small loss (4–10%) of biological activity [unpubl. observations].

#### TCID<sub>50</sub> Assay

H9 cultures (4.5 ml, 0.8 × 10<sup>6</sup> cells/ml) were inoculated with 0.5 ml of four independent serial 10-fold dilutions of inoculated preparations. Cultures were examined twice a week for cytopathic effects, and reverse transcriptase (RT) activity in the supernatant was measured as previously described [19]. A culture was considered to be infected when there was elevated supernatant RT activity (>2 × negative mean) at least on two occasions, and multinuclear giant cells were present. Cultures were followed up to 5 weeks after inoculation. Titres are expressed as TCID<sub>50</sub>, being the reciprocal of the dilution that was able to infect 50% of the inoculated cultures under the conditions described [11].

## Results

### Virus Titre (TCID<sub>50</sub>) Reduction by Lyophilisation

The TCID<sub>50</sub> titre of the HIV-inoculated preparations used in these experiments ranged from 10<sup>7.0</sup> to 10<sup>10.5</sup>. Lyophilisation of these plasma products alone resulted in a reduction of TCID<sub>50</sub> titre of maximally 3.2 logs (table I). Post-lyophilisation titres before heat treatment procedures ranged from 10<sup>4.6</sup> to 10<sup>7.8</sup>. In a series of six experiments, moisture contents in lyophilised preparations (2.5–4.8%) met manufacturing specification (<5%), with the exception of experiment No. 3 (6.5%).

However, TCID<sub>50</sub> reduction by heat treatment in this preparation did not significantly differ from the other experiments (table II).

### Thermal Decay of HIV in Lyophilised Plasma Products at 60°C

Thermal inactivation of HIV-inoculated and lyophilised plasma products was studied by incubation for 8–72 h in a 60.0°C water bath. In four experiments (No. 1–4), due to the high initial titre, residual infectious virus was detectable in samples heated for 72 h, allowing an accurate estimation of TCID<sub>50</sub> reduction after prolonged heat treatment. Sometimes, infection of H9 cultures inoculated with heated plasma products became evident only after 3–4 weeks of culture. Therefore, cultures were followed up to 5 weeks. In these experiments thermal decay of HIV determined by TCID<sub>50</sub> titrations was similar in all four plasma products used as matrix during heat treatment (fig. 1a, b). The kinetics of thermal inactivation tend to proceed according to a biphasic curve, showing 1.5–4.3 logs TCID<sub>50</sub> reduction in the first 8 h of heat treatment and 1.0–2.7 logs TCID<sub>50</sub> reduction in the remaining 64 h. The cumulative TCID<sub>50</sub> reductions (table II) obtained by heat treatment as such for 72 h ranged from 4.2 to 5.6 logs with a mean of 5.0 logs reduction. From table II it is clear that heat treatment for shorter periods (8 or 24 h) resulted in a variable reduction of TCID<sub>50</sub> titres, ranging from 1.5–4.3 logs (8 h) to 2.8–4.3 logs (24 h). No significant differences in TCID<sub>50</sub> titre reductions after 72 h were observed between plasma

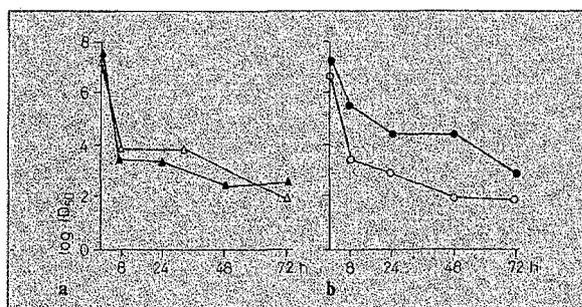


Fig. 1. Thermal decay of HIV in lyophilised blood products. a Small pool cryoprecipitate ( $\Delta$ ), factor VIII concentrate ( $\blacktriangle$ ) and b  $C_1$ -esterase inhibitor concentrate ( $\bullet$ ) and prothrombin complex ( $\circ$ ) were spiked with high titres of HIV, lyophilised and heat-treated at  $60^\circ\text{C}$ .

products inoculated with PEG or ultracentrifuge-concentrated inocula. In addition, the omission of 1% (w/v) sucrose added to factor VIII concentrate as a stabiliser did not have a significant effect on the  $\text{TCID}_{50}$  reduction by heat treatment (data not shown). In two additional experiments (No. 5 and 6) with post-lyophilisation titres  $<10^5$ , a similar biphasic curve was obtained, leaving no detectable virus after 48 and 72 h, respectively. The  $\text{TCID}_{50}$  reduction resulting from both lyophilisation and heat treatment is summarized in table III.

### Discussion

Convincing evidence has been produced by others that HIV is extremely heat labile in a liquid phase [9, 12]. Similar observations were made in our laboratory with HIV-inoculated liquid hepatitis B vaccine [Tersmette et al., unpubl. observations]. Because of the rapid decrease of biological activity of plasma products, especially clotting factor concentrates during heat treatment in the fluid phase, these products must be heat-treated in the lyophilised state. At the present time, data concerning evaluation of heat inactivation of HIV in the lyophilised state are rather limited [12, 13]. In reports published to date, virus titres at the beginning of the heat treatment never exceed  $10^5$  and hence little or no infectious virus could be detected after 24 h of heat treatment [12, 13]. In order to be able to fully evaluate the kinetics and efficacy of the heat inactivation of HIV over a 72-hour time

Table III. Inactivation of HIV in plasma products by lyophilisation and heat treatment

Exp. No.	Virus stock <sup>1</sup>	Matrix	$\text{TCID}_{50}$ before lyophilisation <sup>2</sup>	Residual $\text{TCID}_{50}$ <sup>3</sup>	Overall $\text{TCID}_{50}$ reduction <sup>4</sup>
1	PEG	small pool cryoprecipitate	$\geq 7.5$	1.9	$\geq 5.6$
2	PEG	factor VIII concentrate	9.0	2.5	6.5
3	PEG	prothrombin complex	9.5	$\leq 2.0$	$\geq 7.5$
4	UC	$C_1$ -esterase inhibitor concentrate	10.5	3.1	7.4
5	UC	prothrombin complex	7.0	$\leq 1.5$	$\geq 5.5$ <sup>5</sup>
6	UC	prothrombin complex	7.6	$\leq 0.5$	$\geq 7.1$

<sup>1</sup> Virus concentrated by PEG or ultracentrifuge pelleting.

<sup>2</sup> Expressed as log value.

<sup>3</sup>  $\text{TCID}_{50}$  after lyophilisation and 72 h,  $60^\circ\text{C}$ ; expressed as log value.

<sup>4</sup> Expressed as  $-\log P_t/P_0$ .

<sup>5</sup> Minimal  $\text{TCID}_{50}$  reduction as assessed after 48 h of heat treatment.

period, we aimed at the highest achievable virus titre in the inocula. In the present study in four experiments, lyophilised plasma products containing  $\text{TCID}_{50}$  titres of  $10^{6.8}$ – $10^{7.8}$  HIV were prepared and subsequently heat treated. With these materials a biphasic thermal decay of HIV was observed. After a  $\text{TCID}_{50}$  reduction of 1.5–4.3 logs in the first 8 h of heat treatment, additional inactivation was observed in the remaining inactivation period. In all four experiments a considerable  $\text{TCID}_{50}$  reduction of  $\geq 4.2$  logs was found after 72 h of heat treatment.

The biphasic inactivation may be explained in two ways: First, it could be due to a heterogeneity in the virus inoculum. However, whereas genomic *in vitro* variation of the virus has never been observed [14], two different varieties of HIV were shown to be no less sensitive to heat treatment [12, 13]. Moreover, a similar biphasic curve was found in other experiments on viral inactivation in lyophilised preparations. This biphasic curve did not change upon a second consecutive inactivation [15]. More likely, the inactivation kinetics may be attributed to an inhomogeneous distribution of virus particles in the lyophilised matrix, resulting in differential heat sen-

sitivity. In our experiments the shape of the inactivation curve was not influenced by the titres of the inocula. Experiments with post-lyophilisation titres  $<10^5$  showed a similar initial thermal decay leaving no detectable virus after prolonged heating. Hence, our results imply that the TCID<sub>50</sub> reduction in the initial 8–24 h of heat treatment does not allow an extrapolation with respect to reduction obtained by prolonged periods of time, as has been done by others [12].

Taken together, lyophilisation and heat treatment resulted in a mean TCID<sub>50</sub> reduction of at least 6.6 logs (range  $\geq 5.5$ –7.5 logs) (table III). In preliminary clinical studies on the efficacy of heat treatment, as investigated by studying seroconversion rates of haemophiliacs treated with either heat-treated or conventional factor VIII preparations, no seroconversions could be recorded in patients treated with heat-inactivated preparations [16–18]. Recently, two cases of possible transmission of HIV with heat-treated factor VIII concentrate have been reported [19, 20]. However, in both cases a preparation heated only for 30 h had been administered [21]. In a hypothetical worst case scenario, it has been deduced that a commercial factor preparation produced from unscreened plasma pools may contain  $2 \times 10^5$  TCID<sub>50</sub>/ml HIV at most [22]. If so, inactivation procedures that result in a reduction of more than 5.3 logs in infectivity may be considered to prevent the transmission of HIV. Our findings, in combination with the majority of the clinical reports available, strongly suggest that this assumption is valid, and that transmission of HIV and AIDS can be adequately prevented by heat inactivation procedures as described in this paper.

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