HIV INACTIVATING PROCEDURES IN BLOOD PRODUCTS

Introduction

The first case of transfusion-associated AIDS was described in 1982 in an infant after receiving a transfusion for erythroblastosis fetalis (1,2). The first 3 cases of AIDS in patients with hemophilia were reported in 1982 after treatment with lyophilized factor VIII concentrate (3). Recognition of the occurrence of transfusion-associated AIDS resulted in great concern for the safety of the blood supply. Each lot of lyophilized Factor VIII concentrate is prepared from plasma from up to 22 thousand plasmapheresis donors (4). Primarily of AIDS concern, in early 1983 producers of clotting factor concentrates began a voluntary self-deferral program in order to screen out high risk groups. Obviously, such screening was far from 100% effective. The identification of the AIDS virus by Montagnier (5) and Gallo (6) in 1984 led to development of HIV antibody screening tests and test kits were licensed in early 1985 (7). Thus, beginning in the spring of 1985, all blood and plasma collected in the US is being tested for HIV antibody.

The identification of the AIDS virus also resulted in studies of methods of inactivating the virus. At the present time, there are a number of commercially available clotting factor concentrates where a variety of viral inactivation procedures have been employed (8-18).

It is the purpose of this review to discuss and to examine the clinical efficacy of HIV inactivating procedures used in plasma products.

Thermal inactivation of HIV

A significant portion of retroviruses withstand the procedures used to prepare factor VIII. Retroviruses are resistant to freeze drying and can be kept in a lyophilized form for at least one year without a decrease in titer. The lyophilization process was found to cause a reduction of infectious virus titer of only about 10-100 fold (20).

The sensitivity of HIV to heat has been reported by several groups (19-24). At the request of the National Hemophilia Foundation Medical and Scientific Advisory Council, the Center for Disease Control (CDC) conducted thermal stability studies with the AIDS virus. Preliminary data of the CDC group in September 1984 formed the scientific basis for the subsequent recommendation that heat-treated lyophilized antihemophilic Factor VIII and Factor IX concentrates be used in the treatment of hemophilia (25). Recent extensive CDC data supported this recommendation (23).

Methods and Materials

Virus and cell lines

Most of the investigators used either human T-cell leukemia line, H-9 or HIV was propagated in normal phytohemagglutinin (PHA) stimulated lymphocytes. Aliquotes of virus suspension were stored in liquid nitrogen. Virus inocula used in the most studies had infectivity titers of $10^5$ (24).

Viral infectivity assays

In vitro systems for the determination of inactivation of the HIV virus have been developed. These systems depend on the ability of this virus to infect normal lymphocytes in culture system. Infectivity is determined by either the amount of reverse transcriptase activity of viral particles in the supernate of the cultured lymphocytes, or by indirect immunofluorescence assay or by antigen-capture enzyme-linked immunosorbent assays (20,22). These systems are made more sensitive by using serial dilutions of virus inoculum such as the ID50 assay (23). The ID50 can be defined as the infectious viral titer that is the reciprocal of the dilution (in a log scale) at which 50% of the cultures are positive for virus. The ID50 assay has been employed by researchers at the CDC Atlanta, to determine the effectiveness of thermal inactivation of the AIDS virus.
Dry versus wet heat HIV inactivation

Heat has been applied to clotting factor concentrates in various ways with different combinations of time and temperature exposure (19-24). Rates of thermal inactivation of HIV varied with temperature and differed in liquid state as compared to dry lyophilized virus suspensions (23). Multiple studies have confirmed the resistance of the lyophilized retroviruses to heat treatment as opposed to the heat-sensitive liquid phase (20, 23).

In CDC study HIV virus was added to liquid factor concentrate, which was then processed according to the standard manufacturing procedures and heated either wet or lyophilized, dry (23). The original inocula, preheated samples, and prepared final product were evaluated as to in vitro viral infectivity. These researchers determined that the thermal decay/kill curve showed a first-order rate constant, and that one log dilution, termed D10, of virus was inactivated in 30 seconds when liquid F VIII was heated at 60°C. When virus was inactivated in lyophilized F VIII, one log dilution was inactivated in 30 minutes. (Fig. 1)

In general, the range of the temperature and time exposure used to inactivate HIV in lyophilized form are from 60°C for 20 hours to 68°C for 72 hours. HIV in the liquid phase of F VIII concentrate required only 56°C for 1 hour for complete viral inactivation (19, 20). As the minimum heating time of "dry" product is 60°C for at least 10 hours, the extrapolated viral kill would be a minimum of 20 logs, which should provide a wide safety margin. (4).

Significance of the initial virus titer in heat HIV inactivation

Levy et al. have found that the initial virus titer differences may reflect small variations in virus survival. High titer retrovirus ($10^{15}$) lyophilized preparation showed very low levels of residual infectious particles after heat treatment at 60°C for 72 hours (19). Levy et al. experiments have shown that heating lyophilized F VIII for 72 hours at 68°C or the liquid product for 10 hours at 60°C will eliminate infectious HIV if it is not present in the plasma at more than $10^6$ infectious particles per ml (20).

Influence of stabilizers on heat HIV inactivation

Thermal inactivation of HIV in factor concentrates requires inclusion of stabilizers, such as sugars, amino acids and salts to reduce unwanted protein denaturation and loss of biological activity. Levy et al have found that in the absence of sucrose, wet heat HIV inactivation was significantly more potent (20). Horowitz et al. reported that the rate of virus inactivation at 60°C was decreased by at least 100 to 700 fold by inclusion of 2.75 M glycine and 50% sucrose or 3.0 M potassium citrate (21). The CDC study, however, has not confirmed significant differences in thermal decay of HIV in unstabilized liquid F VIII concentrate or that stabilized with 50% sucrose and 2M glycine - D10 = 30 sec. versus 32 sec. respectively (23).

Hemostatic efficacy of heated factor concentrates

Heat-treated factor concentrates have been available for clinical use in the United States for approximately 3 years. The hemostatic efficacy of heated factor concentrates has been extensively studied. Recovery and survival studies of F VIII have been carried out on all commercial products available in the United States (Table 1) (8-12). These studies have had a similar design in which small numbers of hemophiliac volunteers have been infused usually in a double-blind format with both heated and untreated products. The crossover to the alternate infusate was done at a minimum of 7 days. The recovery and survival data all show approximate equivalence under the experimental conditions for both F VIII and F IX concentrates. Delaunay et al. found no significant difference in the F VIII plasma recovery or plasma half-life of the factor (8). Horowitz et al. found no loss of F VIII activity in lyophilized preparation after 72 hours of heating at 60°C (21). The heat-treated concentrates appeared bioequivalent to the untreated concentrates with the additional benefit of inactivation of potentially present infectious viruses. Nevertheless, there are anecdotal reports in the literature of hemorrhagic episodes not responding to heat-treated product, necessitating more careful investigation of clinical efficacy of heat-treated factor preparations (4).
Theoretical risks of heat HIV inactivation

There are a number of theoretical risk problems connected with the use of heat-treated factor concentrate preparations. These include:

1. Failure of the primary objective of viral inactivation
2. Altered hemostatic efficacy
3. Denatured proteins resulting in:
   a. Anaphylactic or other immune reactions
   b. Immune complex mediated disease
   c. Neoantigen exposure resulting in inhibitor stimulation
   d. Reticuloendothelial overload of denatured protein

Studies were carried out in an attempt to detect protein biochemical changes and denaturation both at the in vitro level and by animal studies. Double immunodiffusion in agarose gels using antisera raised in rabbits to heated and non heat-treated F VIII failed to identify any neoantigens on cross-reactivity, thus confirming their immunological identity. Experiments investigating protein denaturation have failed to reveal protein changes on SDS-polyacrylamide gel electrophoresis, gel permeation chromatography and emission fluorescence spectra (26-29).

Animal studies have shown equal tolerance to the infusion of heated and non-heated concentrates (26-29).

Thus, preliminary studies such as double immunodiffusion and animal studies failed to reveal neoantigen formation or protein changes, indicating a substantial safety margin.

HIV seroconversion after use of heat-treated clotting factor concentrates

The heat-treated clotting factor concentrates have been used clinically for over 3 years in the United States and for a longer period in Europe (4). Thirteen major non-U.S. Hemophilia treatment centers (Europe, Canada and Australia) were asked to provide information concerning HIV seroconversions occurring in patients receiving heat-treated concentrates only. No cases of seroconversion following the use of donor-screened, heat-treated products were identified through this survey (30). Rouzioux et al followed a group of 18 hemophiliacs who were treated exclusively with heat-treated products. None seroconverted over a period of at least 6 months (31). However, there have been two separate reports of single patients, previously documented as seronegative, becoming seropositive after infusion with heat-treated unscreened serum products (32,33). Such reports indicate that despite both in vitro and in vivo data, longer followup is required for the conclusion as to the efficacy of HIV inactivation by the heat-treatment process.

Chemical HIV inactivation

Chemical processes for HIV inactivation in plasma and its fractionated products have been also evaluated in the literature (16,17,24,33-36).

Chemicals have been known to exert their antiviral action at different targets of the viral structure. Thus, lipid solubilizing agents, such as ethyl ether and Triton X-100 detergent would be expected to inactivate envelope viruses. The surface active agents, such as Nonoxynol-9 and Amphotericin B act at the membrane level.

DNA polymerase inhibitors, such as retinoic acid and suramin will inhibit HIV replication.

Lipid solubilizing agents inactivating HIV in vitro

An NIH group investigated HIV inactivation by lipid solubilizing agents, such as chloroform, ethyl-ether, formaldehyde, betapropiolactone, Triton X-100 and by ultraviolet light.
in the presence of psoralen (Table II) (24). Their results indicate that a variety of chemical procedures are effective in reduction of HIV infectivity.

**Surfactants inactivating HIV in vitro**

**Nonoxynol-9** is a non-ionic surfactant, a chemical ingredient of several spermicides. A West Germany group described *in vitro* inactivation of HIV by nonoxynol-9 at concentrations of 0.05% or more. This experiment suggests that commercially available spermicides containing 1% nonoxynol-9 may have a protective effect against AIDS infection (37).

Recently, **amphotericin B methyl ester**, which is known to be active against a variety of lipid-enveloped RNA and DNA viruses, was reported to be active against HIV (38). The binding of amphotericin B to cholesterol in the membrane of cells causes changes in cell permeability and function, and its binding to sterols of lipid-enveloped viruses causes loss of their infectivity (38).

**Peptide-T** is a synthetic analog of an octapeptide, receptor like pattern of antigen T4, derived from neuropeptide in human brain, which potently inhibits HIV at a concentration of 100nM *in vitro* (35).

**Inhibitors of reverse transcriptase inactivating HIV in vitro**

**Suramin** is a drug, used in the therapy of Rhodesian trypanosomiasis, known to inhibit the reverse transcriptase of a number of retroviruses. Suramin was the first drug reported to have an *in vitro* inhibitory effect on HIV replication (34).

The effect of **retinoic acid** on HIV replication *in vitro* was investigated in Japan. Concomitant treatment of the cells with 5ug/ml of retinoic acid and various concentrations of suramin resulted in the more effective inhibition of HIV replication than suramin alone (35).

There are several commercially available factor concentrates that underwent HIV chemical inactivation.

1) **Betapropiolactone** treated factor IX prothrombin complex concentrates are presently commercially available in Europe (marketed by Biotest) and are currently under trial in the United States (4).

2) **Betapropiolactone-ultraviolet light procedure** has shown encouraging *in vitro* data with greater than 3.0 log HIV inactivation. Betapropiolactone causes, however, extensive protein denaturation with some loss in potency of the prothrombin-complex proteins (4,13,14). It is noteworthy that HIV is not inactivated by ultraviolet radiation alone in doses much higher than those usually employed in the laboratories of operating rooms. ( )

3) The organic solvent **tri(n-butyl)phosphate** has been used together with the detergents **Tween-80** or sodium cholate by researchers at the New York Blood Center to inactivate contaminating viruses. These agents are added either early or partway through the fractionation process for 6 hours. Sodium cholate is the detergent used in the F VIII process and Tween-80 in the F IX process. In both systems, the added agents are removed from the processed proteins by a chromatographic step (16,17,32).

**Elimination of HIV during preparation of immunoglobulins**

Recently, the safety of immunoglobulin and hyperimmunoglobulin preparations have been the subject of discussions (42,43). Preparations containing antibodies to HIV and possible causative agents of AIDS have been major concerns.

The CDC and FDA after testing lots of immunoglobulin produced between 1982 and 1985 found that, although the results were positive for HIV antibodies, they were not infectious. Studies indicate that recipients of these immunoglobulin preparations did not later seroconvert to HIV positivity (44).

Additionally, recent CDC evaluation of the Cohn-Oncey cold-ethanol fractionation
method determined that the process is effective in eliminating HIV infectivity. Reduction of infectious retrovirus titers were $10^5$ to $10^8$-fold through Cohn-Oncley cold ethanol fractionation from plasma to fraction II, $10^3$ to $10^5$-fold through incubation at pH 4.0 and another $10^4$ fold through incubation of the purified liquid immunoglobulin preparations at 27°C or 45°C (45). The results support the clinical and epidemiological evidence that therapeutic immunoglobulin preparations do not transmit AIDS virus.

**Conclusion**

1. Multiple independent studies confirmed that thermal procedures of HIV inactivation provide large but not absolute margin of safety and expected efficacy of plasma preparations. Use of heat-treated products will reduce AIDS transmission by antihemophilic factor concentrates.

2. Chemical HIV inactivation procedures, described in the literature, are mostly *in vitro* experiments. Further information is required to define chemical HIV inactivation applicable to blood products.

3. Cohn-Oncley cole ethanold fractionation immunoglobulin preparation procedure is effective for elimination of infectious retroviruses. No transmission of AIDS has so far been observed after administration of specific gammaglobulin.

4. Most of the work with chemical and physical agents has been focussed on inactivation of HIV in plasma and not in cellular blood products. No information is available on HIV inactivation in whole blood and cellular blood components.
REFERENCES


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11. Stagnaro T. Personal communication.


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# TABLE I
Viral inactivation procedures and recovery of Factor VIII

<table>
<thead>
<tr>
<th>Product</th>
<th>Method of viral inactivation</th>
<th>Percent recovery F VIII</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Heat, dry, 60°C, 72 hr.</td>
<td>95</td>
</tr>
<tr>
<td>Hyland Travenol (9)</td>
<td>Heat, dry, 68°C, 72 hr.</td>
<td>100</td>
</tr>
<tr>
<td>Cutter Biological (12)</td>
<td>Heat, dry, 60°C, 30 hr.</td>
<td>74</td>
</tr>
<tr>
<td>Armour (10)</td>
<td>Heat, dry, suspended in n-Hepatane, 60°C, 20 hr.</td>
<td>89</td>
</tr>
<tr>
<td>Alpha Therapeutics (8)</td>
<td>Steam, under nitrogen 60°C, 10 hr. or 80°C, 1 hr.</td>
<td></td>
</tr>
<tr>
<td>Immuno-Vienna (18)</td>
<td></td>
<td></td>
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# TABLE II
Chemical HIV inactivation (24)

<table>
<thead>
<tr>
<th>Inactivation method</th>
<th>Duration</th>
<th>Infectivity IVIU/ml</th>
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<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>&gt;10^6</td>
</tr>
<tr>
<td>Chloroform</td>
<td>15 min.</td>
<td>&lt;10^0</td>
</tr>
<tr>
<td>Ethyl ether, 4°C</td>
<td>1 hr.</td>
<td>&lt;10^1</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>10^5</td>
</tr>
<tr>
<td>Betapropriolactone 0.1%, 4°C</td>
<td>18 hr.</td>
<td>&lt;10^0</td>
</tr>
<tr>
<td>Formaldehyde 1:1000, 4°C</td>
<td>18 hr.</td>
<td>&lt;10^0</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>10^4</td>
</tr>
<tr>
<td>Triton X-100, 0.5%</td>
<td>1 hr.</td>
<td>&lt;10^0</td>
</tr>
<tr>
<td>Ultraviolet light &amp; Psoralen</td>
<td>2 hr.</td>
<td>&lt;10^0</td>
</tr>
</tbody>
</table>

*IVIU/ml - *in vitro* infectious unit
Figure 1: Thermal decay of HIV in Factor VIII preparations at 60°C

- HIV in liquid factor VIII concentrate  D10 = 30 sec.
- HIV in lyophilized dry factor VIII concentrate  D10 = 30 min.