Thermal Inactivation of the AIDS Virus, HTLV-III/LAV, with Special Reference to Antihemophilic Factor

J. S. McDougal, L. S. Martin, S. P. Cort, M. Mozen, C. M. Heldebrant, and B. L. Evatt

Division of Host Factors, Center for Infectious Diseases, Centers for Disease Control, Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA; Cutter Laboratories, Berkeley, CA; and Research and Development Department, Alpha Therapeutic Corporation, Los Angeles, CA.

Address reprint request to J. S. McDougal, M.D., Immunology Branch, I-1202, Centers for Disease Control, Atlanta, GA 30333.

Running Title: Thermal inactivation of the AIDS virus
Summary

The virus that causes the acquired immunodeficiency syndrome (AIDS), human T lymphotropic virus/lymphadenopathy associated virus, (HTLV-III/LAV), was incubated at temperatures from 37° - 60°C and virus titer (ID-50) was determined over time by a microculture infectivity assay. The rate of thermal decay was consistent with first-order kinetics, and these data were used to construct a linear Arrhenius plot (r=0.99) which was used to determine inactivation time as a function of temperature. In the liquid state, thermal decay was little affected by matrix (culture media, serum, or liquid Factor VIII). In the lyophilized state, the time required to reduce virus titer 10-fold (1 log) at 60°C was 32 minutes compared to 24 seconds in the liquid state. HTLV-III/LAV in liquid Antihemophilic Factor VIII or IX was lyophilized and heated according to commercial manufacturers' specifications. Infectious virus was undetectable with these regimens. Heat treatment should reduce or stop transmission of HTLV-III/LAV by commercial Antihemophilic Factor VIII or IX.
Introduction

Transmission of the acquired immunodeficiency syndrome (AIDS) by plasma products is a major concern, particularly to the hemophilia community (1). At the request of the National Hemophilia Foundation Medical and Scientific Advisory Council, we conducted thermal stability studies with the AIDS virus (human T lymphotropic virus type III or lymphadenopathy associated virus, HTLV-III/LAV). Preliminary data presented to the Advisory Council at the Centers for Disease Control (CDC) in September 1984 formed the scientific basis for their subsequent recommendation that heat-treated lyophilized Antihemophilic Factor VIII and Factor IX concentrates be used in the treatment of hemophilia (2). We now report more extensive data that support this recommendation.

Methods

HTLV-III/LAV was propagated in normal phytohemagglutin (PHA)-stimulated lymphocytes which were cultured in RPMI media (Gibco, Grand Island, NY) containing 10% v/v fetal calf serum (FCS, Gibco) and 10% v/v interleukin-2 (IL-2, Cellular Products, Buffalo, NY) as previously described (3). Culture supernate was harvested when virus levels had peaked as determined by reverse transcriptase (RT) activity and immunoassay for virus antigen (3). Supernates were sequentially centrifuged at 300 x g for 7 min and 1500 x g for 20 min and stored in 0.5- to 1.0-ml aliquots in liquid nitrogen. Virus inocula used in these studies had infectivity titers of >10^5.

The microculture assay for the titration of infectious HTLV-III/LAV has been described in detail (3). Briefly, PHA-stimulated normal human
lymphocytes are inoculated with serial 10-fold dilutions of virus inoculum and incubated for 18 h at 37°C. The cells are then washed and plated in microculture (10-20 cultures per dilution; 1 x 10^5 cells per culture in 0.25 ml of media). Every 3 days, 100 µl of supernate is removed and replaced with fresh media. The supernates are then assayed for virus by an antigen capture assay (3). Infectious virus titer (ID-50) is defined as the reciprocal of the dilution at which 50% of the cultures are positive for virus (3,4). Previously established (3) features of the infectivity assay system relevant to the interpretation of the thermal inactivation experiments include the following: The antigen capture assay is as sensitive as the RT assay for detecting supernate virus (3). The ID-50 assay, in turn, is several orders of magnitude more sensitive than either assay for detecting small amounts of virus (3). In the ID-50 assay, active virus replication is required to obtain a measurable titer. After incubation with virus, the infected cells are washed, and any antigen carryover from the inoculum is insufficient to register a positive virus capture assay result (3).

For thermal inactivation of virus in a liquid matrix, aliquots of the virus inoculum (HTLV-III/LAV in RPMI media containing 10% FCS and 10% IL-2) were incubated at the temperatures indicated. Exposure times were staggered such that all incubations were terminated at the same time. Aliquots were placed in an ice bath and diluted in media, and ID-50 titrations were performed.

Virus inocula were shipped on dry ice to several manufacturers of Factor concentrates. They mixed 1 part inoculum with 9 parts liquid Antihemophilic Factor, lyophilized the material, and heated it. They then returned the freeze-thawed virus inoculum, prelyophilization specimens, postlyophilization specimens, and post heat-treatment specimens to CDC on dry ice. Uninoculated Antihemophilic Factor preparations, which were processed in parallel, met the
manufacturers' specifications for potency (Factor activity) and residual moisture analysis. Factor activity is reduced 10% - 40% by heat treatment.

At CDC, specimens were reconstituted to the original volume, allowed to stand at room temperature for 30 min, and then serially diluted for ID-50 titrations. As indicated in the text, some specimens were further evaluated with sequential subculture at days 12-14 and 22-24 with fresh PHA-stimulated lymphocytes; these specimens were monitored for virus replication for a total culture period of 30-36 days.

Results

Thermal inactivation of HTLV-III/LAV in a liquid matrix was performed on a virus inoculum with an ID-50 titer of $>10^5$ (Fig. 1). For each temperature studied, a plot of the natural logarithm of the ID-50 (expressed as the fraction of original ID-50, C/Co) versus time was, by linear regression, a straight line with r values of $>0.94$. The only exception was the 50°C plot ($r=0.87$). These data are consistent with first order kinetics. The slopes of the thermal decay curves (plotted as $\ln C/Co$ versus t) are the first-order rate constants, which were used to construct an Arrhenius plot ($\log_{10}$ first-order rate constant versus $1/T$ in degrees Kelvin) with an r value of 0.99 (Fig. 1). The D10 values beside the Arrhenius plot indicate the time required to reduce the virus titer 10-fold (1 log). D10 values for the 37°C, 45°C, 50°C, 56°C, and 60°C temperatures are measured values. The D10 values at 65°C and 70°C are extrapolated from the Arrhenius plot. The increment in temperature that results in a 10-fold change in D10 (the Z value) was 5.34°C.

The thermal decay of virus in a matrix of 90% normal human serum at 56°C was similar to that of virus in culture media (D10 of 117 sec and 121 sec, respectively). The thermal decays at 60°C of an HTLV-III/LAV inoculum in
liquid Factor VIII (D10=22 sec) and liquid Factor VIII stabilized with 50% sucrose and 2 M glycine (D10=30 sec) were similar to that of virus in culture media (D10=24 sec) (Fig. 2). The thermal decay of virus lyophilized in Factor VIII was more prolonged (D10=32 min) (Fig. 2). ID-50 data were also derived on virus inocula that were lyophilized with Factor VIII or Factor IX and heat-treated according to manufacturers' protocols (Table 1). We could not detect virus in any of the heated preparations, i.e., no cultures at the first dilution tested were positive nor was virus detected by prolonged subculture with fresh PHA-stimulated lymphocytes. Twenty-six virus-inoculated products from 11 different lots were lyophilized and treated at 60°C or 68°C for 20-72 h. Virus was undetectable in all of them.

Discussion

HTLV-III/LAV is very heat labile. In a liquid matrix, there is very little difference in HTLV-III/LAV thermal decay when virus is present in culture media, serum, liquid Factor VIII, or Factor VIII containing stabilizers (Figs. 1 and 2). Some degree of protection from thermal inactivation is found in the lyophilized state compared with the liquid state. However, compared with the stability of lyophilized functional proteins like antibodies or enzymes (for which half-lives at temperatures of 50°-60°C are measured in days), lyophiilized virus is quite heat labile (Fig. 2, Table 1). Our results indicate that HTLV-III/LAV has comparable or somewhat greater heat lability than the mouse xenotropic type C retrovirus which was studied by Levy et al. (5). Our results are also similar to those of Spire et al. who reported the decay in RT activity at 56°C of an HTLV-III/LAV inoculum in the liquid state (6).
The ID-50 assay has several advantages for this type of study. First and foremost, an estimation of virus infectivity is needed. The ID-50 assay is measured over a greater range than the 1- or 2-log range found with the RT or antigen capture assay. Finally, the ID-50 assay is several orders of magnitude more sensitive for small amounts of virus than is either the RT or antigen capture assay (3). However, the relationship between the lower limits of sensitivity for infectious virus in this assay and infectivity in man is unknown. The only information we have bearing on this is that one chimpanzee inoculated with a dose of 1120 ID-50 developed viremia within 3 weeks; another chimpanzee given the same inoculum at a dose of 0.1 ID-50 has not developed viremia or seropositivity over a 6 month follow-up.

No one has reported the isolation of HTLV-III/LAV from Antihemophilic Factor using macroculture isolation techniques despite compelling evidence that this material transmits AIDS (1). It is likely that our assay does not measure the critical infectious dose of virus. No assay can discriminate one from zero infectious virus particles, and we are forced to rely on observed log reduction in the measurable range and extrapolation of thermal decay data to make inferences about efficacy of decontamination. Certainly, a procedure that reduces titer only 1- to 2-logs is insufficient to decontaminate Antihemophilic Factor because this reduction is obtained with lyophilization alone, and lyophilized products transmit AIDS. However the first order kinetics of thermal decay and the long heating times, which are based on considerations for hepatitis virus inactivation (7), indicate an expected reduction of 37 logs at 60°C in 20 h (and even greater for higher temperatures and times). This should provide a large, if not absolute, margin of safety. A preliminary clinical report supports the efficacy of heat treatment. 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 compared with 5 seroconversions in 29 matched hemophilia controls treated with conventional product (8). Thus, both in vitro and vivo data indicate that the use of heat-treated products will reduce (and hopefully terminate) AIDS transmission by Antihemophilic Factor.
References


Table 1. Thermal Inactivation of HTLV-III/LAV in Lyophilized Factor Concentrates

<table>
<thead>
<tr>
<th>Concentrate</th>
<th>Pre-lyophilization</th>
<th>Post-lyophilization</th>
<th>Heat</th>
<th>Post-Heat</th>
</tr>
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<tbody>
<tr>
<td>Factor VIII</td>
<td>$10^6.20$</td>
<td>$10^4.27$</td>
<td>$68^\circ C, 24h$</td>
<td>undetectable*</td>
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<tr>
<td>Factor IX</td>
<td>ND*</td>
<td>$10^3.74$</td>
<td>$68^\circ C, 24h$</td>
<td>undetectable*</td>
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<td>Factor VIII</td>
<td>$10^5.26$</td>
<td>$10^3.68$</td>
<td>$60^\circ C, 20h$</td>
<td>undetectable*</td>
</tr>
<tr>
<td>Factor IX</td>
<td>$10^5.25$</td>
<td>$10^4.40$</td>
<td>$60^\circ C, 20h$</td>
<td>undetectable*</td>
</tr>
</tbody>
</table>

* no measurable ID-50, no positive cultures, and no virus detected in 2 sequential subcultures with fresh PHA-stimulated lymphocytes which were monitored for a total of 30-36 days.

+ ID-50 of HTLV-III/LAV-inoculated liquid Factor IX was not determined, however, the virus preparation used for inoculation had an ID-50 at $>10^6$. 
Figure Legends

Fig. 1. **Thermal inactivation of HTLV-III/LAV in a liquid (culture media) matrix.** ID-50 is expressed as the fraction of preincubation ID-50 (C/Co) and plotted on a log_{10} scale (1 log increment represents a 10-fold loss in ID-50). ID-50 determinations for the 37°C and 45°C incubations were also performed at 4, 8, and 24 hours (data not shown). Lines were constructed by linear regression. From a plot of the natural logarithm of C/Co versus time, the slopes (1st-order rate constants) for each temperature were calculated, and an Arrhenius plot was constructed. D10 values indicate the time required for a 10-fold reduction in virus titer at the indicated temperatures. Closed circles are experimentally measured data points. Open circles are extrapolated from the Arrhenius plot.

Fig. 2. **Thermal decay of HTLV-III/LAV in Factor VIII preparations at 60°C.** HTLV-III/LAV in reconstituted (liquid) Factor VIII concentrate, \( \bullet \); HTLV-III/LAV in liquid Factor VIII containing 50% sucrose and 2 M glycine, \( \circ \); HTLV-III/LAV added to liquid Factor VIII and lyophilized, \( \Delta \). Virus was not detected in the liquid preparations after the 2 min time point. Virus was detected in the lyophilized preparation at 120 min but only in the 1st dilution where 3 of 10 cultures were positive. Thereafter, virus was undetectable.