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Safety of Human Blood Products: Inactivation of Retroviruses by Heat Treatment at 60°C (42045)

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Abstract. Acquired immune deficiency syndrome (AIDS) can be transferred to patients by blood transfusions or human blood preparations, such as cryoprecipitates or factor VIII concentrates. Retroviruses have been discussed as infectious AIDS agents and more recently human T-lymphotropic retroviruses designated as HTLV type III and LAV (lymphadenopathy-associated virus) have been isolated from AIDS patients. Whether heat treatment at 60°C (pasteurization) of liquid human plasma protein preparations inactivates retroviruses was therefore investigated. Pasteurization had already been included in the routine manufacturing process of human plasma protein preparations in order to guarantee safety with regard to hepatitis B. Since high titer preparations of human retroviruses were not available, heat inactivation was studied using Rous sarcoma virus added to the various plasma protein preparations tested. This retrovirus which was obtained in preparations of 6.0 $\log_{10}$ FFU/ml was shown to be at least as heat stable as two mammalian retroviruses studied, i.e., feline and simian sarcoma virus. In all of eight different plasma protein preparations tested, Rous sarcoma virus was completely inactivated after a heat treatment lasting no longer than 4 hr. It is thus concluded that pasteurization of liquid plasma protein preparations at 60°C over a period of 10 hr must confer safety to these products with respect to AIDS, provided that the AIDS agents are retroviruses of comparable heat stability as Rous sarcoma virus and the mammalian retroviruses tested.

About 3 years ago the acquired immunodeficiency syndrome (AIDS) was described as a new disease (1) observed in homosexual men with multiple sexual partners, intravenous drug abusers, hemophiliacs, blood transfusion recipients, and those with close heterosexual contacts of members of these high risk groups (2, 3). The epidemiology of the spreading of this disease has led to the assumption that it might be caused by an infectious agent which because of the decreased number of T-helper lymphocytes in AIDS patients seems to have the capacity of infecting human T lymphocytes. In 1983 a human retrovirus, the human T-cell leukemia virus type I (HTLV-I), was discussed as a possible candidate for being an infectious AIDS agent (4), but the data were not convincing. In the meantime a disease closely resembling human AIDS was found in rhesus monkey herds named "simian AIDS" (SAIDS) (5). By successfully infecting rhesus monkeys it was shown that SAIDS is caused by a type D retrovirus (6). Furthermore, recently published data have provided compelling evidence for HTLV-I related human retroviruses being the infectious AIDS agents (7, 8). These retroviruses, designated as lymphadenopathy-associated virus (LAV), and HTLV type III (HTLV-III), respectively, have been isolated from AIDS patients in France and in the United States. These viruses may be closely related or even identical. HTLV-III has frequently been found in AIDS and pre-AIDS patients. Almost 100% of AIDS patients tested have antibodies against HTLV-III (9).

Since according to accumulating data retroviruses are strong candidates for being AIDS agents and since this disease can be transferred by blood products, particularly

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by clotting factor preparations (10, 11), we decided to study whether retroviruses can be efficiently inactivated by heat treatment of liquid human plasma protein preparations. Heat treatment of the liquid preparations at 60°C (pasteurization) had previously been introduced into the manufacturing procedure of clotting factor VIII concentrates (F VIII) and other plasma proteins in order to inactivate hepatitis B virus, the most dangerous infectious agent for hemophiliacs in untreated F VIII preparations. Since none of the above mentioned AIDS or SAIDS retrovirus candidates were at our disposal and since these viruses are secreted into the supernatant of cell cultures only at low titers, we decided that from the large number of animal retroviruses available to us we would select a retrovirus for these heat inactivation studies which could be grown to high titers and is among the most stable representatives of this group. The virus chosen was Rous sarcoma virus (RSV). Here, we compare the inactivation kinetics at 60°C of RSV and two mammalian retroviruses and then report on the inactivation of RSV in a series of liquid plasma protein preparations.

Materials and Methods. *Virus and infectivity assays.* The Schmidt-Ruppin strain of Rous sarcoma virus, subgroup A (RSV) was quantitated in a focus test essentially as developed by Rubin (12) as modified by Vogt (13) using chicken embryo cells in the third or fourth passage. Simian sarcoma virus (SiSV) was assayed in a focus test using the MFS-4 marmoset fetal skin strain, and Snyder-Theilen feline sarcoma virus (FeSV) in a focus test utilizing CCL-64 mink cells.

Plasma protein and heat treatment. The human plasma proteins studied were F VIII, antihemophilic cryoprecipitate (AHC), antithrombin III concentrate (AT III), clotting factor IX concentrate (F IX), clotting factor XIII concentrate (F XIII), prothrombin-complex concentrate (PPSB), Cl esterase inhibitor concentrate (Cl INH), and fibrinogen concentrate. These proteins are routinely used for the therapeutic treatment of congenital or acquired deficiencies of either the blood clotting or fibrinolytic system. For the retrovirus inactivation study samples were taken from a production lot before the heat treatment of this lot was performed (14). These samples are sterile—achieved by 0.2-μm filtration—and thus do not contain any blood cells or cell debris. After addition of saccharose and glycin as stabilizers these samples were mixed with a RSV preparation (9:1), thoroughly stirred, and then incubated in a water bath at 60°C over the indicated time. This heat inactivation procedure considerably differs from the heat treatment of the final lyophilized products as performed by other manufacturers. For virus titration samples were taken after thorough mixing, but before heat treatment, and during heat treatment as indicated in Table I.

At the end of heat treatment all samples were assayed simultaneously for infectious virus. In those experiments in which the heat stability of FeLV, SiSV, and RSV was compared, these viruses were heat treated in Dulbecco's minimal essential medium.

Results. *Heat stability of retroviruses.* As shown in Fig. 1 the retroviruses RSV, FeSV, and SiSV are of comparably low stability at 60°C, because all three viruses are inactivated under these conditions within less than 60 min. Of course, the time needed for complete inactivation is directly related to the titer of the virus sample. Since for the experiments with the plasma proteins the RSV preparations used had to be diluted 10-fold, a RSV preparation of a higher titer than 4.0 $\log_{10}$ FFU/ml was needed. Such a preparation having a virus titer of 6.0 $\log_{10}$ FFU/ml was diluted in the plasma protein preparations and then subjected to heat treatment. In the plasma protein preparation RSV could not be completely inactivated in 1 hr, but a prolonged treatment was required to inactivate all infectious virus.

Inactivation of RSV in blood products. The results of heat inactivation of RSV in the liquid preparations of eight different plasma proteins are summarized in Table I. While in most of these experiments complete RSV inactivation was achieved after a period of 2 hr, in AHC and PPSB low amounts of virus were still detectable after this time. Complete inactivation of RSV in these preparations could, however, be demonstrated after a 4-hr period. Whether this is a significant difference depending on the individual blood product or whether this falls within the range of the experimental system used cannot be

TABLE I. INACTIVATION OF ROUS SARCOMA VIRUS IN VARIOUS LIQUID PLASMA PROTEIN PREPARATIONS BY HEAT TREATMENT AT 60°C

Plasma protein	Virus titer (FFU/ml) after treatment at 60°C over various periods of time (hr)				
	0	0.5	1	2	4
1. Experiment					
F VIII	5×10^4	2×10^1	4×10^0	0[a]	0
AT III	5×10^4	1.5×10^2	4×10^1	0	0
Fibrinogen	9×10^4	3×10^2	4×10^1	0	0
2. Experiment					
Medium	3.5×10^5	n.d.[b]	4×10^1	0	0
AT III	3.5×10^5	n.d.	7×10^1	0	0
Fibrinogen	2×10^5	n.d.	3×10^2	0	0
AHC	3×10^5	n.d.	4×10^2	4×10^1	0
C1 inactivator	3×10^5	n.d.	4×10^1	0	0
F XIII	2×10^5	n.d.	1×10^2	0	0
F IX	2.5×10^5	n.d.	1×10^2	0	0
PPSB	3×10^5	n.d.	4×10^2	1×10^1	0

[a] 0 = no infectious virus detectable in a 1-ml sample; supernatants of negative cell cultures were subcultivated consequently three times to prove that no infectious RSV had been in the original sample.
[b] n.d. = not done.

clearly answered. Since heat treatment as included in the manufacturing procedure consists of an incubation at 60°C for 10 hr, we decided against a detailed investigation of the significance of these slight differences.

In the case of F VIII we also determined the inactivation time of Rous-associated virus, using an assay based on endpoint dilutions

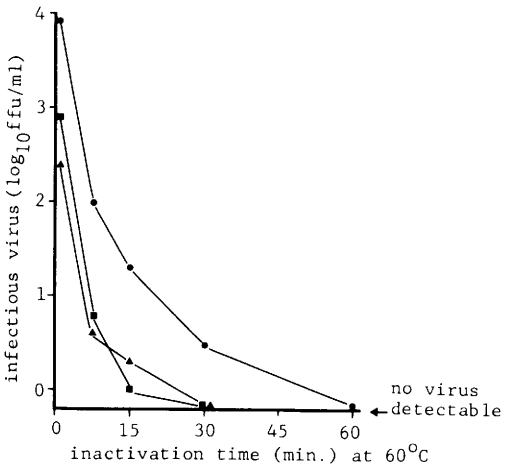

FIG. 1. Kinetics of inactivation of the three different retroviruses, Rous sarcoma virus (●), feline sarcoma virus (■), and simian sarcoma virus (▲) by heat treatment at 60°C suspended in maintenance medium.

indicated by a reverse transcriptase test after 14 days cultivation. We found that this virus when used in a preparation of 5.0 $\log_{10}$ FFU/ml was completely inactivated within 2 hr just as RSV (data not shown).

Discussion. In the past hemophiliacs easily contracted hepatitis B from crude F VIII concentrates which at the same time, however, were life-saving therapeutics for these patients. Although the amount of hepatitis B virus in F VIII preparations can be considerably reduced by using HBsAg negative blood donations only, and by employing efficient methods for the concentration and purification of this plasma protein, it cannot be guaranteed that F VIII preparations, even when HBsAg negative, do not contain any infective hepatitis B virus. HBsAg negative means that no HBsAg is detectable by means of the most sensitive enzymeimmuno- or radioimmunoassays. Although a HBsAg content of $\geqslant 1$ ng/ml can be excluded by using these assays, such HBsAg negative samples can still contain up to $10^{2.5}$ CID_{50}/ml (CID_{50} = chimpanzee infective dose 50) of hepatitis B virus. A 10-hr heat treatment of the liquid plasma protein preparation was therefore included in the production procedure thus efficiently inactivating residual infectious hepatitis B virus (15). Furthermore, there is

now some good evidence that AIDS occurring in hemophiliacs is caused by an infectious agent transferred by unheated F VIII preparations. Several studies performed during the last year indicate that the infectious AIDS agent might be a human retrovirus. We therefore investigated whether the heat treatment of liquid F VIII preparations at 60°C, which had been developed for hepatitis B virus inactivation could also inactivate retroviruses. The candidate virus RSV used in our studies proved to be as stable as two mammalian retroviruses tested and had the added advantage that it could be propagated to high titers, i.e., the inactivation experiments could be done with retrovirus preparations of 6.0 $\log_{10}$ FFU/ml.

In addition to F VIII we tested seven other plasma proteins in this study for two reasons: (i) we wanted to know whether this procedure with respect to retroviruses is generally effective and (ii) we wanted to exclude the possibility that other plasma proteins which are already on the market or are intended to come onto the market soon could transfer infectious retroviruses after pasteurization. Our data present convincing evidence that this heat treatment procedure can be used as a general method. Moreover, it can also be applied to the inactivation of viruses other than hepatitis B and retroviruses in human plasma protein preparations (manuscript in preparation).

In all plasma protein preparations studied here, complete RSV inactivation was achieved after a 4-hr treatment at most. Since the routine pasteurization procedure consists of a 10-hr treatment at 60°C, there is good reason to assume that this procedure will also be effective even if the human retroviruses causing AIDS should prove to be slightly more stable than RSV or occur in cell-free plasma protein preparations at higher titers than those of RSV used here. Since at present, however, neither LAV nor HTLV III could be isolated from cell-free pre-AIDS or AIDS plasma, it can be assumed that the amounts of these viruses in such plasma samples are rather low.

In summary, we therefore conclude that (i) the conditions of pasteurization as described here conferred safety to all plasma proteins with respect to RSV, (ii) the slight differences in inactivation kinetics as seen among different plasma proteins are of no importance with respect to the long heating period, and (iii) the transfer of AIDS by cell-free plasma proteins can be efficiently prevented by pasteurization provided that the infectious AIDS agent is a retrovirus as published in a series of recent papers (7–9) and that this retrovirus shares the lability to heat treatment with other retroviruses.

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