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Removal and Inactivation of Hepatitis B Virus from Contaminated Pooled Plasma in a Large-Scale Manufacturing Process for Factor VIII and Human Serum Albumin

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Abstract

Background and Objectives: The Japanese Red Cross Society recalled one lot of monoclonal-antibody-purified factor VIII (F VIII) and two lots of human serum albumin (HSA) 5 months after preparation of the final products, because of a procedural error that led to contamination by a unit of plasma positive for hepatitis B surface antigen (HBsAg). We evaluated the effectiveness of virus inactivation/removal in a large-scale process for manufacturing F VIII and HSA. ***Materials and Methods:*** HBV DNA in the retained samples in process was measured by the polymerase chain reaction (PCR). The kinetics of virus inactivation by solvent-detergent (S/D) treatment was examined using model viruses. We also did a look-back survey of the patients who received corresponding products. ***Results:*** Contaminated hepatitis B virus (HBV) DNA became undetectable beyond fraction S IV-I in the albumin process and immunoaffinity chromatography in the F VIII process, respectively. The model viruses were inactivated within 5 s by S/D treatment. There is no evidence that patients were infected by HBV after transfusion of these products. ***Conclusion:*** We conclude that virus inactivation/removal was effectively achieved in a large-scale manufacturing process for F VIII and HSA.

Introduction

The blood supply in our country is safer than ever, because of the use of voluntary donors, screening for the main blood-borne viruses such as hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus types 1 and 2 (HIV-1, HIV-2), and human T cell lymphotropic virus type 1 (HTLV-I), as well as for alanine aminotransferase (ALT) activity. Postscreening safety measures, i.e. virus removal and virus inactivation procedures, are employed with plasma derivatives, such as coagulation factor VIII and human serum albumin (HSA).

However, concern about safety continues and residual risks remain from donation during seroconversion window periods, viral mutation, nonresponder donors, viruses that are not tested for, and procedural error.

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Recently, after a procedural error, one unit of plasma positive for hepatitis B surface antigen (HBsAg) contaminated 14,685 units of pooled plasma. Five months after the preparation of monoclonal-antibody-purified factor VIII (FVIII) and HSA from this pool, we identified this pool as positive for HBsAg. By that time, one lot of F VIII and two lots of HSA had been delivered to hospitals and some of them had already been administered to patients. The remaining products were then urgently recalled. First, we tested for HBV DNA by the polymerase chain reaction (PCR) in the final products, and in samples retained from each step in the manufacturing process. Second, the kinetics of virus inactivation by solvent-detergent (S/D) treatment was examined using Sindbis virus (SIN) and herpes simplex virus (HSV) as models for enveloped virus. Finally, we did a look-back survey of the patients who received these products through collaboration with hospitals all over Japan.

This article gives the results of those validations and of the look-back survey.

Materials and Methods

Source Plasma for the Preparation of HSA and F VIII

Plasma was collected from 14,685 voluntary blood donors qualified by questionnaire in Japanese Red Cross blood centers throughout Japan. All donated blood was screened for HBsAg, anti-HBc, anti-HBs [1], anti-HCV [2], and anti-HIV-1,2 [3] and anti-HTLV-I antibody [4], syphilis and ALT [3]. The plasma separated from these units was sent to the Japanese Red Cross Plasma Fractionation Center and pooled. Except for the one HBsAg-positive unit, which unfortunately contaminated the pool, all other units were qualified by the above screening tests.

Production of HSA and F VIII

After removing cryoprecipitate, HSA was prepared using Cohn's ethanol fractionation procedure [5]. As outlined in figure 1, dissolved P V-2 were concentrated by ultrafiltration and the resulting bulk solution was heated at 60 °C for 10 h for virus inactivation, after which final HSA products were prepared.

Defibrinized solution obtained from dissolved cryoprecipitate was treated by S/D as described by Horowitz et al. [6] for virus inactivation. This S/D-treated solution was then subjected to immunoaffinity column chromatography with anti-F VIII mouse monoclonal antibody, and ion-exchange chromatography, for purification to obtain the final product of F VIII. An outline of the manufacturing process is shown in figure 1. Samples at each step of the fractionation process were retained, kept frozen at –40 °C and later tested for HBV DNA by PCR [7, 8].

Measurement of HBV DNA

From each sample, 100 µl was treated with SDS/proteinase K, and DNA was extracted by phenol/chloroform and purified by ethanol precipitation. PCR was performed by the method described by Iizuka et al. [7]. Quantitation of HBV DNA was carried out by the limiting-dilution method. The sensitivity of this method is 2 copies/100 µl, as determined by branched DNA techniques. The amount of HBV DNA in each sample was calculated as 2 copies/100 µl multiplied by the maximum dilution number in which DNA was detected.

The TaqMan Fluorogenic Detection System [8] was also used for HBV DNA quantitation. HBV DNA extracted with EX-R&D (Sumitomo Metal Industries Ltd., Tokyo, Japan) or the conventional SDS/proteinase K method was amplified and quantified with Prism 7700 (Perkin Elmer Corp./Applied Biosystems, Foster City, Calif., USA). A cloned HBV DNA, the concentration of which was determined based on the absorbance at 260 nm, was used to make a calibration curve. The HBV core region was amplified in TaqMan PCR, the sensitivity of this method being equivalent to the PCR method described above [Hirakawa et al., in preparation].

Virus Inactivation with S/D Treatment

HSV and SIN stock solutions were prepared from the culture supernatant of virus-infected Vero cell. One part of the HSV or SIN stock solution was added to 9 parts of the dissolved cryoprecipitate obtained from our manufacturing process. For virus inactivation with S/D treatment, tri(n-butyl)phosphate (TNBP) and octoxynol 9 (TX-100) were added to bring their concentrations to 0.3 and 1%, respectively. At timed intervals (0 s, 5 s, 10 s, 30 s, 1 min, 10 min and 60 min), aliquots were withdrawn and the inactivation reactions were terminated by mixing with pretreated octadecyl silica resin (WakosilSC18, Wako Pure Chemical Industry, Japan) for 3 min.

HSV infectivity was determined by plaque assay. Tenfold serial-diluted samples were placed on Vero cell confluent cultures in 24-well plates. After 1 h adsorption, the diluted samples were replaced by RPMI-1640 culture medium containing 1% carboxymethyl cellulose. At 3 days after inoculation, the Vero cell cultures were stained with Gentian violet B solution and the number of plaques was counted. Virus infectivity was expressed as plaque-forming units (PFU/ml). SIN infectivity was determined by end-point dilution assay. Tenfold serially diluted samples were made in culture medium (RPMI-1640) and each diluted sample was used to inoculate five replicate wells of cells in 96-well plates. The Vero cell was also used as the indicator cell in this assay. Seventy-two hours after inoculation, virus-induced cytopathology was scored and a tissue culture infectious dose ($TCID_{50}$) value was calculated by the Reed-Muench method.

Look-Back Survey of Patients Who Received F VIII and HSA

To date, we have investigated HBsAg, anti-HBs, anti-HBc in 126 patients who received these F VIII or HSA products, through collaboration with 69 hospitals.

Results

Inactivation and Removal of HBV DNA in the Course of F VIII Manufacturing Process

The amount of contaminating HBV was 2×10^3 copies/ml in the cryoprecipitate solution, and 2×10^2 copies/ml in the defibrinized solution. HBV DNA was still detected in the S/D-treated defibrinized solution (2×10^2 copies/ml) but HBV DNA was not detected in the immunoaffinity chromatography eluate and in the F VIII final product (fig. 1).

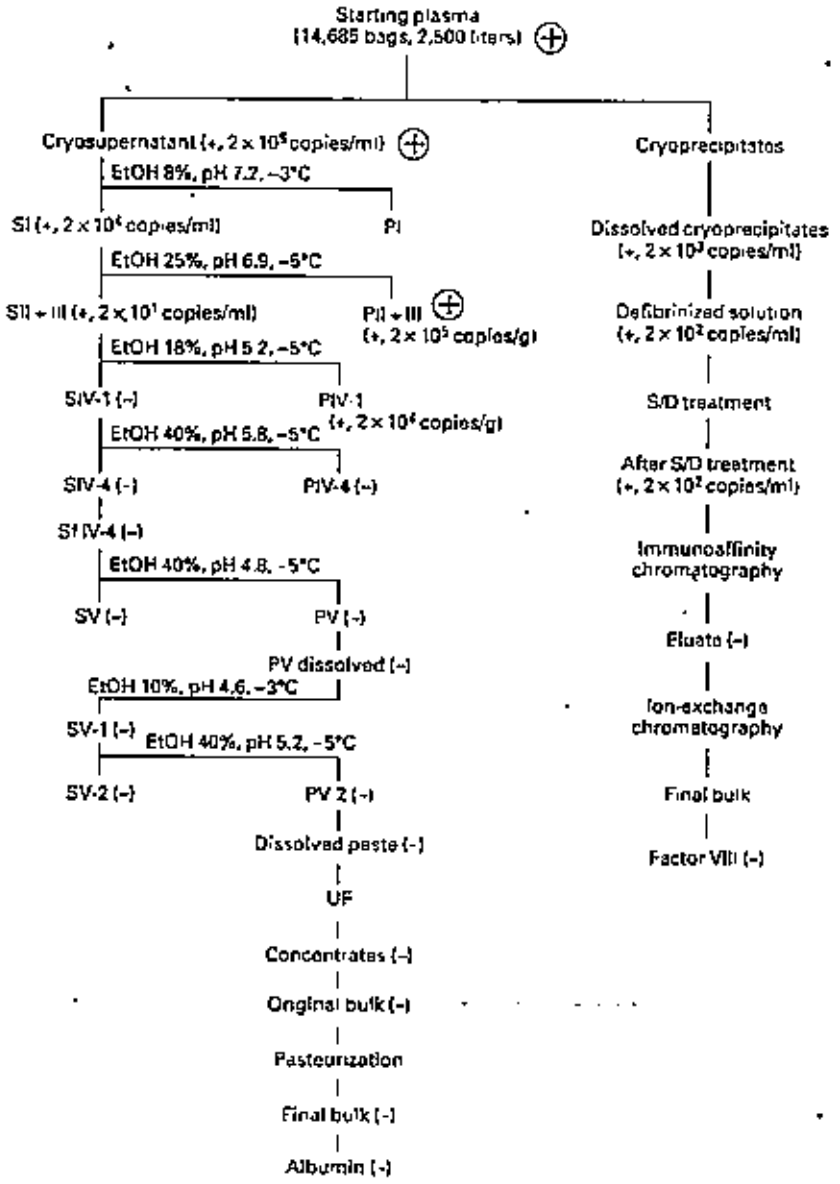
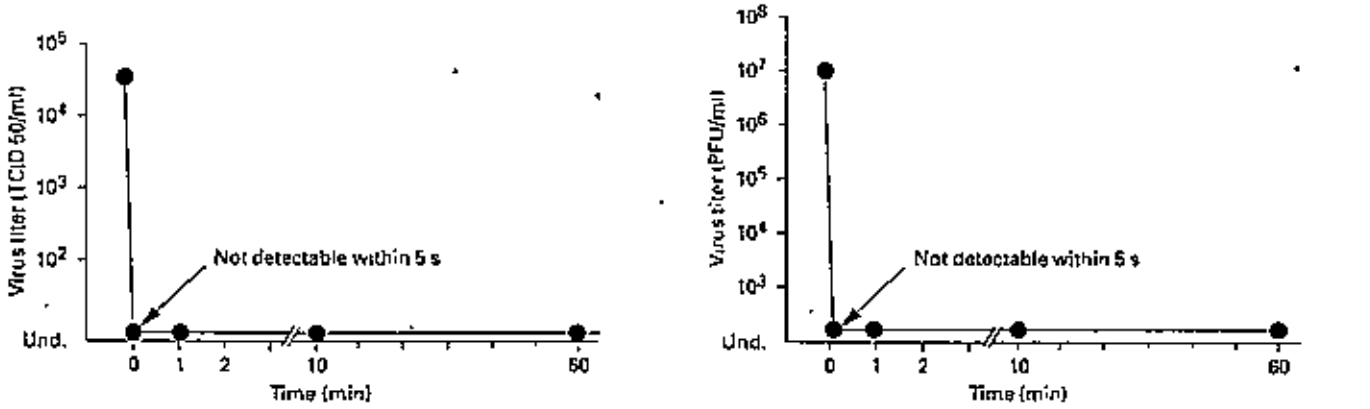


Fig. 1. Process of albumin and F VIII preparation. HBV DNA was measured by PCR. (+) = PCR-positive and number of HBV DNA copies; (−) = HBV DNA undetectable by PCR; ⊕ = HBsAg positive by EIA (Auszyme II®, Abbott).

These data indicated that HBV DNA was effectively removed with the immunoaffinity chromatography washing step.

Removal of HBV DNA in the Course of the HSA Manufacturing Process

HBV DNA in cryosupernatant and samples from each of the following steps in Cohn's ethanol fractionation were measured by PCR. P I, P II+III, P IV-1, P IV-4, P V and P V-2 were dissolved in 3- to 10-fold PBS and dialyzed against PBS. S I, S II+III, S IV-1, S IV-4, Sf IV-4, S V, dissolved P V, and S V-1 and S V-2 were dialyzed against PBS before PCR. The dialyzed samples were subjected to PCR. The amount of HBV DNA was 2×10^5 copies/ml in the cryosupernatant plasma, 2×10^4 copies/ml in S I, 20 copies/ml in S II+III, 2×10^5 copies/g in P II+III, and 2×10^4 copies/g in P IV-1.



a b

Fig. 2. Kinetics of inactivation of SIN (**a**) and HSV (**b**) with S/D treatment. $10^{4.6}$ $TCID_{50}$/ml of SIN V and $10^{7.2}$ PFU/ml of HSV were added to cryoprecipitate dissolved solution, followed by addition of TNBP (0.3%) and octoxynol 9 (1%), and incubated at room temperature. Und: undetectable.

Table 1. Seroconversion and elevation of serum ALT levels of the patients who received corresponding lots of albumin and F VIII

	HBsAg (+)		Anti HBs (+)		Anti HBc (+)		Elevation of ALT	
	pre	post	pre	post	pre	post	pre	post
Albumin	0/33	0/33	0/12	0/12	0/3	0/3	0/28	0/28
F VIII	0/3	0/3	0/1	0/1	–	–	0/3	0/3

However, HBV DNA was not detected in S IV-1, SIV-4, P IV-4, SfIV-4, SV, dissolved PV, S V-1, S V-2, P V-2, dissolved P V-2, the concentrated solution after ultrafiltration, the bulk solution, the bulk solution after heat treatment for 10 h at 60°C, and the final HSA.

Inactivation of Model Virus Infectivity by S/D Treatment

The kinetics of inactivation of SIN and HSV as models of enveloped virus by S/D treatment were investigated. Results are shown in figure 2; $10^{4.6}$ $TCID_{50}$/ml of SIN and $10^{7.2}$ PFU/ml of HSV became undetectable within 5 s, respectively.

Look-Back Survey

The look-back study on 115 patients who received HSA and 11 patients who received F VIII was started after we discovered the contamination by the HBV-DNA-positive unit, and was done retrospectively with the collaboration of 69 hospitals all over Japan. An average of 2.1 months after administration, sera from all these patients gave negative results for HBsAg.

We also had serum taken before administration from 33 of these patients who received albumin and 3 who received F VIII. Testing those, we could examine seroconversion of HBV markers and elevation of serum ALT after administration.

As shown in table 1, serological markers of HBV infection of all those patients were negative before and after transfusion. Elevation of serum ALT level after administration could not be observed in 28 patients who received HSA and 3 patients who received F VIII. We have requested the collaboration of all 69 hospitals to inform us of any of these patients developing HBV infection. So far, we have not yet received any report of HBV infection from the hospitals. Therefore there is no evidence that any of the patients were infected by HBV after transfusion of these lots of HSA and F VIII.

Discussion

An unfortunate procedural error led to a unit of HBsAg-positive plasma contaminating a plasma pool of 14,685 units used for the preparation of F VIII and HSA. After an urgent recall of these plasma derivatives, we carried out a retrospective investigation for HBV DNA by PCR in the retained samples from each step of the fractionation procedure.

First, we demonstrated that HBV DNA was removed after Cohn's fractionation step to separate S IV-1 from P IV-1. On the other hand, 2×10^5 copies/g HBV DNA were found in P II+III, which is raw material for globulin preparation [9–11].

HBsAg was positive by ELISA test (Auszyme II, Abbott) in starting plasma, cryosupernatant, and P II + III. Although P IV-1 was not tested for HBsAg because no sample had been retained, the above results correlate with the reports by Schroeder et al. [9], Berg et al. [10] and Trepo et al. [11]. More interestingly, distribution of Dane particles in F I, F III and F IV, as reported by Trepo [11], correlated well with the distribution of HBV DNA by PCR as described (fig. 1).

We then realized that Cohn's ethanol fractionation procedure, established in 1944, not only purifies plasma proteins but also removes contaminating HBV. After this procedure, HBV DNA was undetectable in all fractions and the final HSA was pasteurized at 60°C for 10 h. Pasteurized albumin has been used since 1948 [12] and for about 50 years there has not been any report of HBV transmission [13].

Second, contaminating HBV DNA was undetectable after purification of F VIII by immunoaffinity chromatography on S/D-treated solution which contained 200 copies/ml HBV DNA. This DNA came from HBV after S/D treatment and the process did not affect detectability of HBV DNA by PCR as reported by Hilfenhaus et al. [14]. However, S/D-treated products have been approved for routine use in numerous countries, including Argentina, Australia, Austria, Belgium, Canada, Czechoslovakia, Denmark, Finland, France, Germany, Israel, Italy, Japan, Korea, the Netherlands, Norway, Poland, Portugal, Saudi Arabia, South Africa, Spain, Sweden, Switzerland, the United Kingdom, the United States, and Venezuela. Notably, the 3.8 million doses of F VIII transfused represents over 45,000 man-years of treatment, assuming an average infusion of 80,000 IU per man-year. Based on current usage patterns, approximately two thirds of the F VIII transfused in North America, western Europe, and Japan is S/D treated. Throughout this time period, not a single case of HBV, HCV, or HIV transmission has been reported [17]. Based on such numerous evidence, HBV DNA in an S/D-treated solution is inactivated HBV, without infectivity.

Griffith [15] described similar results in small-scale spiking experiment to separate virus by immunoaffinity chromatography. Here, we have made the demonstration in a large-scale fractionation procedure. After this, an eluate was applied to an ion-exchange column and HBV DNA was, of course, undetectable in the final product of F VIII. It is well known that when 10^4 chimpanzee infectious doses HBV suspended in factor VIII preparations were treated with TNBP/sodium cholate and inoculated intravenously into 2 chimpanzees, neither showed evidence of hepatitis or HBV infection over 40 weeks of follow-up [16]. Also, no single case of HBV, HCV or HIV transmission has been reported from administration of S/D-treated F VIII concentrates from 1985 through 1993 [17].

The efficacy of S/D treatment was validated by using SIN and HSV as model enveloped viruses. Within 5 s, $10^{4.6}$ $TCID_{50}$/ml of SIN and $10^{7.2}$ PFU/ml of HSV became undetectable. As Griffith [15] described, a model virus VSV in F VIII was inactivated by 3 min S/D treatment. In similar conditions, we describe here the inactivation kinetics of SIN and HSV. These model viruses had no infectivity after 5 s of S/D treatment, confirming the efficacy of inactivation of enveloped viruses. These data support the assertion that contaminating HBV in the present case must have been inactivated by S/D treatment.

The look-back study was started an average of 2.1 months (maximum 4 months) after exposure, as we had to survey retrospectively. If we could have done a prospective study, as we did before [1], we could have observed the HBV makers and serum ALT level every 1 or 2 weeks for more than 3 months. The situation in this accident was different and our look-back limited.

All 141 patients examined after the administration were HBsAg negative. Fortunately, we were able to test pre-administration sera from 33 patients who received albumin and 3 patients who received F VIII, and we could check for seroconversion of HBV markers and elevation of serum ALT levels after the administration.

Since the average window period of HBV infection is estimated to be 59 days [18], negative HBsAg, lack of seroconversion of anti-HBs and anti-HBc and lack of elevation of serum ALT level after administration, we could conclude that HBV infection did not occur in these patients. Thereafter, we have not received any case report of hepatitis B among the 141 patients from 69 collaborating hospitals.

Although the clinical data are not complete, after examining all the information obtained from the hospitals, we could find no evidence of HBV infection after administration of these albumin and F VIII fractions.

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