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Pasteurization as an Efficient Method to Inactivate Blood Borne Viruses in Factor VIII Concentrates

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Summary: *Heat treatment at 60 °C for 10 h in solution (pasteurization) was introduced into the manufacturing process of factor VIII concentrate (Haemate P) in order to considerably reduce the risk of transmission of human pathogenic viruses to haemophiliacs. The results of experimental and clinical studies with regard to hepatitis B, non-A, non-B hepatitis, acquired immune deficiency syndrome (AIDS) and herpes virus infections are reviewed. From this data it is concluded that pasteurization of factor VIII results in a product which is safe with regard to these viral infections. Furthermore, it was shown that pasteurization does not form new antigenic determinants on the factor VIII molecule and compared with the native product does not alter the physiological properties of this protein in patients. In comparison to these advantageous properties of the pasteurized product a slight loss of coagulant activity seems to be acceptable. This loss of yield, however, does not influence the quality or the amount of factor VIII in the final container used for the therapy of haemophilia A patients.*

Zusammenfassung: *Pasteurisierung, eine wirksame Methode zur Virusinaktivierung in Faktor VIII-Konzentraten*

Hitzebehandlung bei 60 °C über 10 h in wäßriger Lösung (Pasteurisierung) wurde in den Produktionsprozeß des Faktor VIII-Konzentrates Haemate P aufgenommen, um die Übertragung humanpathogener Viren auf Hämophile zu verhindern. Die Ergebnisse experimenteller und klinischer Studien im Hinblick auf die Inaktivierung von Hepatitis B, Non-A/Non-B-Hepatitis, Aquired Immune Deficiency Syndrome (AIDS) und Herpes-Virus-Infektionen werden dargestellt. Diese Daten belegen, daß die Pasteurisierung von Faktor VIII-Konzentrat zu einem virussicheren Präparat führt. Weiterhin konnte gezeigt werden, daß durch das Verfahren keine neuen antigenen Determinanten auf dem Faktor VIII-Molekül erzeugt werden und im Vergleich zu dem nativen Produkt die physiologischen Eigenschaften des Proteins nicht verändert werden. Ein geringer Verlust an koagulatorischer Aktivität durch das Pasteurisierungsverfahren beeinflußt die Qualität des Faktor VIII-Konzentrates nicht, da entsprechende Reinigungsschritte eingeführt wurden.

Key words: *Factor VIII concentrates, pasteurization · Haemate P · Viruses, blood borne, inactivation*

1. Introduction

Human plasma protein preparations containing factor VIII are essential life-saving pharmaceutical products for haemophilia A patients, although they bear a potential risk of transmitting virulent human viruses to the recepient [1–3]. Viruses most frequently associated with such plasma preparations are those causing post-transfusion hepatitis (hepatitis B or non-A, non-B hepatitis) and more recently those causing the acquired immune deficiency syndrome (AIDS). In order to prevent the transmission of post-transfusion hepatitis great effort was made to select safe plasma donations and to eliminate viral contaminants by high purification of the factor VIII concentrates. Preselection of the plasma donations was achieved by the use of a sensitive "third generation" hepatitis B surface antigen (HBsAg) assay to exclude hepatitis B virus. The efficient purification methods developed to remove most of the burden of ineffective human plasma proteins were also able to remove any detectable HBsAg which might be used as marker antigen for hepatitis B virus (HBV) and possibly other viral contaminants. Nevertheless, it turned out that such improved factor VIII concentrates still caused post-transfusion hepatitis [4, 5]. Moreover, it was recently proved that factor VIII concentrates can infect haemophiliacs with the AIDS-retrovirus as shown by specific antibodies against this virus which occur at a high rate in factor VIII treated haemophiliacs [3, 6].

Consequently, it was necessary to include a method for virus inactivation into the manufacturing process in addition to preselection of individual plasma donations and of efficient purification procedures. Although a variety of effective methods for virus inactivation were known of from the development of inactivated viral vaccines, here only those could be considered which inactivate viruses but do not or at least not considerably affect the biological activity of the factor VIII molecule. Furthermore, the method chosen must not alter the antigenic sites of the molecule in order not to lead to the formation of new antigenic sites (neo-antigens) as may happen when chemical inactivation methods are used. Not to generate neo-antigens is most important because by the induction of antibodies against neo-antigens in haemophiliacs, an eventually efficacious therapy might be prevented by the rapid elimination of this altered factor VIII molecule in the patients. Several groups therefore searched for a method which could be used as an optimal compromise, i. e. a method 1. conferring the greatest possible rate of inactivation on potentially transmitted infectious agents, 2. only slightly reducing the biological activity of factor VIII if at all, and 3. preventing the formation of neo-antigens.

The method we used was heat treatment of the purified product in solution at 60 °C for 10 h (= pasteurization), a method which was developed more than 40 years ago to destroy the virus of serum hepatitis, hepatitis B virus (HBV), in human serum albumin preparations (reviewed in [7]). In contrast to the fairly heat stable albumin molecule, the more heat labile factor VIII needed high concentrations of glycine and sucrose to stabilize its biological activity. The production procedure of this pasteurized factor VIII concentrate*) (F VIII P) – as well as some data on the virus inactivation by this method – have previously been published elsewhere [8]. Here we review all data on the safety of F VIII P gained from experimental viral studies as well as from a follow-up clinical trial.

2. Human viruses of potential risk in factor VIII concentrates

In principle, all human viruses occurring not cell-bound in the blood of an infected donor can be considered as a potential risk for the recipient of a factor VIII concentrate. Unfortunately, for none of the blood donors it can be excluded that their blood is free of every infectious agent at the time of donation. The same holds true for plasma pools used for the production of factor VIII concentrates. In practice, however, the number of viruses to be considered can be reduced either to those which have been shown to cause infectious diseases in haemophiliacs after treatment with factor VIII concentrates or at least to those which may cause such diseases after transfusion of whole blood [1–3, 9]. As can be seen from Table 1 this number of human pathogenic viruses

Table 1: Viruses potentially occurring in human plasma derivatives.

Virus	Clinical relevance	Virus used in inactivation studies
HBV	infectious agent of hepatitis B	HBV subtype adw
NANB-Viruses	infectious agents of Non-A/Non-B-hepatitis	Hutchinson-Pool[1]
HTLV III, LAV	retrovirus causing AIDS	HTLV III Rous sarcoma virus
CMV EBV HSV	human herpesviruses causing severe diseases in immunocompromised patients	CMV strain Davis EBV-B95-8 HSV-1

LAV = lymphadenopathy-associated virus; CMV = cytomegalo virus; EBV = Epstein-Barr virus; further abbreviations see text.
[1] Virus preparation (National Institute of Health, Bethesda, MD, USA) eliciting NANB-hepatitis in chimpanzees.

is much smaller than that of pathogenic viruses which may occur in the blood under the conditions of an acute infection. This may be explained by the fact that human plasma pools contain immunoglobulins and thus a sufficiently high antibody titer exists against many viruses [10, 11] which can completely neutralize the viruses of one or even some infected plasma donations in a pool consisting of several hundred donations. There is no doubt that HBV and viruses causing non-A, non-B hepatitis are the most important agents, followed by the recently isolated human retrovirus which causes AIDS. In addition, we added three human herpes viruses to this list which have been described as being transmitted by transfusion of whole blood and which play a role as secondary infectious agents in AIDS [12–14]. Also noted in Table 1 are the representatives for each group, which were studied in in vitro or in vivo experimental systems. We did not, however, consider any infectious agents other than viruses because the manufacturing process includes at least one filtration through a membrane filter of a pore size of 0.22 μm which eliminates all non-viral infectious agents from the final product.

*) Haemate P; manufacturer: Behringwerke AG, Marburg/Lahn (Federal Republic of Germany).

3. Efficiency of pasteurization on hepatitis B and non-A/non-B hepatitis viruses

Depending on the dose and the number of different production lots of factor VIII concentrates used for the treatment of haemophiliacs, up to 100% of them showed diagnostic parameters of hepatitis B and more than 50% experienced a hepatitis B or non-A, non-B hepatitis [1, 2, 4]. Through the development of the so-called "third-generation" HBsAg test method for the detection of HBV carriers, plasmas of those donors containing detectable amounts of HBsAg were excluded from plasma pools used for factor VIII concentrate production. This method, however, could not prevent the transmission of hepatitis B [5]. In addition as a possible consequence of the exclusion of HBsAg positive plasma donations non-A, non-B hepatitis was diagnosed more frequently in haemophiliacs [15]. Since at present no specific serological test exists for the in vitro diagnosis of this hepatitis and transaminase screening is not sufficient, the exclusion of blood donations from non-A, non-B hepatitis virus (NANB virus) carriers is not possible [16, 17]. It was therefore most desirable to investigate the efficiency of pasteurization on the inactivation of HBV as well as NANB virus.

It is a pity, however, that the testing of the infectivity and thus the testing of the inactivation of HBV as well as NANB virus is limited, because the only test system is the chimpanzee. Due to the shortage of these animals titrations of virus samples in a large number of animals or detailed studies of the efficiency of various steps of the manufacturing process regarding virus elimination are not possible. We therefore used HBsAg as a parameter of HBV elimination under the conditions of F VIII P purification, assuming that HBsAg would behave as HBV because both have a similar surface structure and are of comparable size at least when compared with the factor VIII molecule. To prove the efficiency of HBV elimination plus consecutive inactivation, infectious virus was added to a cryoprecipitate sample before purification. Then this sample, a sample after purification but before pasteurization and a sample of the final pasteurized product were tested for infectious HBV in chimpanzees [8, 18, 19]. The results of this experiment are summarized in Table 2 and show that more than 90% of HBsAg are eliminated by purification alone and that the remaining infectious HBV not eliminated by purification was completely inactivated by pasteurization.

Table 2: Elimination of biologically active hepatitis B virus by purification method and pasteurization of liquid factor VIII.

Material tested	HBsAg (ng/ml)	Infectivity in chimpanzees hepatitis pos./animals inoculated
Cryoprecipitate	17	4/4
Purified F VIII concentrate	≤ 1	4/4
Purified F VIII concentrate, heat treated (F VIII P)	≤ 1	0/7

Although these experiments demonstrate the efficiency of the manufacturing process regarding hepatitis B we must be aware of the fact that HBV is quite heat stable as published by Shikata et al. who showed that by pasteurization no higher inactivation rate than approximately 10^4 can be achieved [20]. Thus heat stability of HBV again requests the combination of using HBsAg negative plasma, an efficient purification procedure and heat treatment for virus inactivation. To prove the safety of F VIII P manufactured this way a long term surveillance study was performed in cooperation with six hemophilia centers ([21], manuscript in preparation). 31 previously untreated haemophiliacs, with no signs or symptoms of liver disease, receiving F VIII P were treated and kept under control for a minimum of six month up to a maximum of 60 months (average 17 months). During the

[redacted] of this study each patient received an average dose of 8,750 units of F VIII P equalling a total amount of 1,892,270 units of 186 routine production lots. None of the patients acquired a hepatitis B, one patient developed low antibody titers against HBsAg and HBcAg but no rise of serum transaminases. Since this patient had received nearly 380,000 units of F VIII P of a total number of about 1,000 applications it might be argued that this patient has been immunized with inactivated, yet antigenically intact viral particles present in a certain number of the F VIII P preparations used. Whatever the reason might be, these results demonstrate a superior safety of F VIII P as compared with an almost 100% seroconversion in haemophiliacs treated with conventional factor VIII preparations [1]. In a similar study none of five haemophilia B patients receiving a pasteurized factor IX preparation developed antibodies to HBV or had any symptoms of liver disease. This again demonstrates the safety of pasteurized plasma proteins.

Compared with HBV, the possibility of investigating the elimination or inactivation of NANB virus is even more limited. We have already mentioned that no specific diagnostic assay exists to detect virus carriers. This is due to the fact that we do not know the causative virus or possibly viruses, although we have plasma samples defined as transmitting NANB hepatitis [22–24]. These human plasma samples obtained from NANB hepatitis patients have been characterized as infectious because after inoculation into chimpanzees they cause raised serum transaminase levels whereby any other viral agent causing hepatitis was excluded [25]. The evaluation of the manufacturing process of F VIII P and its safety can therefore only be studied 1. by adding a certain amount of an infectious human NANB plasma sample to a plasma pool used for the production of F VIII P and by then comparing the infectivity of this mixture with that of the final F VIII P preparation in chimpanzees or 2. by a follow-up of patients frequently treated with F VIII P as the only human blood derivative. Both were done in the case of F VIII P. In the chimpanzee experiment a NANB positive plasma sample containing $10^{5.5}$ CID_{50} (= chimpanzee infectious dose) was added to a purified factor VIII preparation before heat treatment which was then pasteurized and processed to the final F VIII P preparation. The resulting amount of F VIII P, which theoretically contained $10^{5.5}$ CID_{50} was injected into 4 chimpanzees but did not cause any NANB infection, whereas 3 chimpanzees inoculated with the same amout of the non-heat treated mixture developed high transaminase levels indicating infectious NANB virus in this sample (R. Mauler et al., to be published). The follow-up clinical study described above with regard to hepatitis B was also evaluated for increased transaminases regarding NANB hepatitis. 25 out of 31 patients showed normal transaminase levels during the entire observation period, 4 patients showed only moderate elevations not exceeding 120 U/l alanine aminotransferase. Two patients had markedly increased transaminase levels indicating a NANB hepatitis. Although a connection with F VIII P therapy cannot be totally excluded, the fact that both patients acquired the infection following surgery in the same center indicates a post surgical infection ([21], manuscript in preparation). The comparison of the results of this study with the data published by others on the frequency of NANB hepatitis in haemophiliacs treated with unheated factor VIII concentrates, again very impressingly demonstrates the efficiency of pasteurization.

4. Pasteurization and safety from AIDS

Although homosexuals and drug addicts have been found as the groups with the highest risk of being infected with the AIDS agent [26, 27], it was very soon clearly shown that human blood or plasma protein preparations obtained from AIDS patients transmitted the disease to their recipients [28, 29]. In 1983, Montagnier and co-workers described a retrovirus which they had isolated from an AIDS patient as the possible infectious agent (Barré-Sinoussi et al. [30]) and in 1984 Gallo and co-workers published in a series of papers data which proved that such a human retrovirus, designated as LAV (= lymphadenopathy associated virus) by the French group and as HTLV III (= human T-cell lymphotropic virus) by Gallo's group, was indeed the AIDS causing agent [31]. Because at that time no sensitive assay for the titration of HTLV III/LAV existed, we investigated the inactivation of an avian retrovirus, the Rous sarcoma virus (RSV), under the conditions of pasteurization [32]. This virus had been selected for these experiments, because it was at least as heat stable as two mammalian retroviruses tested in comparison and because it could be grown to high titers and hence high rates of virus inactivation could be tested. In these experiments we found that complete inactivation of $10^{4.7}$ ffu (focus forming units) of RSV in a factor VIII preparation was achieved after a 2-h heat treatment. Recently, we were able to show that HTLV III is considerably less heat stable than other retroviruses, particularly RSV as can be seen from the inactivation kinetics of HTLV III in comparison with RSV and two other mammalian retroviruses at 60 °C in isotonic buffer solution or culture medium (Fig. 1). In addition, recent data on the inactivation of HTLV III in F VIII P under the conditions of the pasteurization process indicates that HTLV III is also easily inactivated under these conditions [33, 34]. We therefore conclude that AIDS causing human retroviruses are most efficiently inactivated by the 10-h heat treatment of the pasteurization process. There is no doubt today that with regard to this group of viruses pasteurized factor VIII concentrates – even when produced from HTLV III/LAV positive blood – must be free of any infectious retrovirus and thus unequivocally safe regarding AIDS.

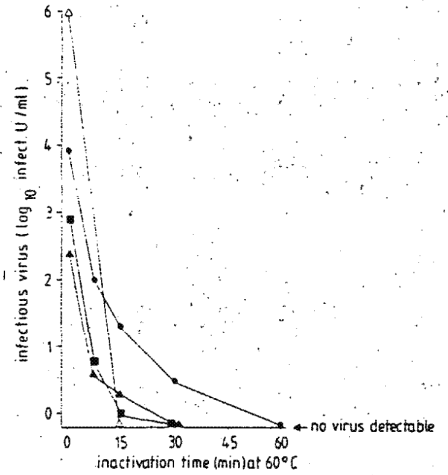

Fig 1: Kinetics of heat inactivation (60 °C) of four different retroviruses. Rous sarcoma virus (●), feline sarcoma virus (■), and simian sarcoma virus (▲) suspended in cell culture medium, and human T-lymphotropic virus type III (HTLV III, Δ) in isotonic buffer solution.

Nevertheless, it might be of interest to investigate whether those haemophiliacs treated with F VIII P preparations differ significantly regarding an anti HTLV III/LAV antibody response compared to those treated with non-heat treated factor VIII concentrates. The results of such a retrospective study have recently been published [35] and show that none of the patients treated with F VIII P developed anti-HTLV III antibodies, whereas in this study as well as in other studies [3, 6, 36] the majority of haemophiliacs treated with unheated factor VIII concentrates were seropositive (Table 3). On the basis of these serological findings it can be concluded that many of the conventional factor VIII concentrates are contaminated with HTLV III/LAV. The fact that even none of the patients treated with F VIII P developed anti HTLV antibodies could have at least two reasons: 1. the amount of non-infectious HTLV III/LAV antigen injected

[redacted]: Anti-HTLV III antibodies in haemophiliacs substituted with different factor VIII concentrates.

Treatment	Duration (yr) of treatment with F VIII P[1]	Median annual dose (IU)	Frequency of anti-HTLV III
Conventional F VIII	–	68.000	60/100 (60%)
Conventional F VIII, then continued with F VIII P	3	16.500	3[2]/15 (20%)
F VIII P only	3	3.700	0/18 (0%)

[1] Mean duration ranging from 2–6 years.
[2] These three patients were already positive before change of treatment.

with F VIII P may not suffice to induce antibodies when given by the for this purpose very inproper intravenous route or 2. more probably all HTLV III/LAV antigen had been removed from F VIII P by the very efficient purification procedure used.

5. Inactivation of other human viruses by pasteurization

No other human viruses can be compared in importance to those causing hepatitis or AIDS. Nevertheless, we decided to extend our studies to those viruses which have been described as playing a role as opportunistic infections in AIDS patients [13, 14, 37] or as being transmitted by whole blood transfusion [9]. These viruses are three members of the herpesvirus group, namely cytomegalovirus (CMV), Epstein-Barr virus (EBV) and herpes simplex virus (HSV). They normally do not develop viraemia in infected individuals and when found in the blood then only or predominantly cell-bound and they can thus not be transmitted by a cell-free plasma protein preparation. We could show in F VIII P that each of these herpesviruses when added as cell-free virus particles to purified factor VIII before heat treatment were efficiently inactivated by pasteurization [34]. The different inactivation rates (see Table 4) depend on the infectivity

Table 4: Inactivation of various viruses by heat treatment at 60 °C in solution (pasteurization)[1].

Virus	Inactivation rate achieved and time of heat treatment needed	
	Rate (log 10)	Time (h)
DNA viruses		
Cytomegalovirus	⩾ 6.0	8
Epstein-Barr virus	⩾ 4.6	1
Herpes simplex virus	⩾ 6.0	4
Vaccinia virus	6.3	10
RNA viruses		
HTLV III	⩾ 5.5[2]	0.5
Rous sarcoma virus	⩾ 4.7	2
Polio virus type 1	⩾ 6.6	8

[1] Factor VIII samples were taken from the manufacturing process before heat treatment, virus was added, the resulting mixture heat treated and aliquots assayed for infectious virus.
[2] This rate includes heat treatment (inactivation rate ⩾ 4.0 $\log_{10}$ after 0.5 h heat treatment) plus reisolation of factor VIII from this mixture by precipitation according to the manufacturing process.

titers of the original virus samples added to F VIII P and are therefore rather low in the case of EBV. It is obvious that to produce high titer preparations of EBV is much more cumbersome than it is for instance of HSV. Again, based on these experimental data it is evident that the pasteurization procedure is very efficient in inactivating viruses. Two other viruses, the poliovirus (RNA virus) and the vaccinia virus (DNA virus), shown in this Table have been included in our investigations as viruses which can be easily tested in a virus laboratory although they do not play any role as a risk factor in factor VIII therapy.

6. Stability of factor VIII to pasteurization

Although F VIII P has been shown to be a safe product, safety is not the most essential property of a factor VIII preparation but the biological activity which enables blood clotting. The crucial question therefore is whether under the conditions of pasteurization the biological activity of the factor VIII molecule is maintained and whether its physiological properties in the patient are not altered. We thus studied 1. the factor VIII C activity during pasteurization, 2. the immunogenicity of F VIII P in rabbits and 3. the half-life and recovery of F VIII P in human patients. The determination of procoagulant activity of a F VIII preparation before and after pasteurization revealed some loss of biological activity which usually was lower than that observed after Tween (polysorbate) 80/ether treatment of factor VIII concentrates [38]. With regard to posssible neo-antigens which might have been formed under the conditions of pasteurization, as recently discussed by Bird et al. [39], we investigated whether rabbits which were repeatedly immunized with F VIII P and thus developed high antibody titers against the native factor VIII additionally formed antibodies which only reacted to F VIII P. We found no such antibodies, i.e. no new antigenic determinants had been formed on pasteurized factor VIII molecules [40]. In addition, the biological half-life and the recovery of F VIII P determined in haemophiliacs, when compared with published results for non-heat treated factor VIII preparations show that F VIII P cannot be distinguished from conventional products and as shown the same holds for pasteurized factor IX (Table 5) [41, 42]. Furthermore, neither higher incidence of inhibitors or decreased tolerance was observed in patients treated with F VIII P.

Table 5: Mean half-life and recovery of F VIII and F IX concentrates.

	Mean half-life (h)	Revovery (%)
F VIII[1]	12 –13	82
F VIII P	12.1 ± 5.06	74
F IX concentrate[1]	18 –38	20–50
F IX P	10.8 ± 35.4	43

[1] Data taken from references [38] and [39].

7. Conclusion

The urgent need to develop factor VIII concentrates further in order to reduce the risk of transmitting viral infections, particularly hepatitis and AIDS, has already been discussed in the beginning of this review. Safety can be conferred by using various inactivation procedures, but one must be aware of the fact that each inactivation method may also have some influence on the biological properties of the factor VIII molecule thus either destroying its clotting activity or changing its physiological behavior in the patient. In fact detailed and laborious experimental work had to be done in order to develop a safe and efficacious factor VIII concentrate. As discussed in this review it was found that efficient purification of HBsAg negative plasma followed by pasteurization of the resulting factor VIII concentrate represents an optimal procedure because 1. it proved to result in a safe product with regard to hepatitis B and NANB hepatitis as shown experimentally as well as in a follow-up clinical trial, 2. it results in a product which does not transmit AIDS because the heat labile AIDS causing retroviruses are most efficiently inactivated, 3. it also inactivates herpes viruses which may, if at all, play a minor role as a risk factor, 4. it does not generate any neo-antigens and 5. the pasteurized product does not differ from the native one regarding pharmacokinetics in the patients. As has been shown the slight loss of coagulant activity of factor VIII does not influence the quality of the product but has to be accepted as the price to be paid for the benefit of a safe product.

In addition, other procedures have been developed [38, 43, 44] to reduce the risk of the transmission of infectious diseases by factor VIII concentrates and at least in one case an excellent experimental study has been performed to demonstrate the efficiency of this procedure regarding hepatitis B and NANB hepatitis [42]. In order to be in a position to

the various procedures used and to compare the efficacy and safety of the resulting product, for each procedure one needs comprehensive data on the inactivation of various viruses, the properties of the resulting product subjected to these procedures and the results of clinical trials regarding viral infections of treated patients and the biological properties of the factor VIII in these patients. It is thus important that all data will be published concerning other factor VIII concentrates subjected to other inactivation methods than pasteurization in order to decide which method and which product might be the best for the sake of the haemophilic patient, i. e. to provide optimal therapy combined with the lowest risk of infection.

8. References

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