Inactivation of 12 Viruses by Heating Steps Applied During Manufacture of a Hepatitis B Vaccine

P.N. Lelie, H.W. Reesink, and C.J. Lucas

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam, Amsterdam, The Netherlands

The efficacy of two heating cycles (90 sec at 103 °C and 10 hr at 65 °C) used during manufacture of a plasma-derived hepatitis-B vaccine was validated for the inactivation of 12 virus families. A period of 15 min warming up to 65 °C had already completely inactivated representatives of nine virus families, i.e., poxvirus (vaccinia), picornavirus (encephalomyocarditis virus), togavirus (sindbis virus), coronavirus (mouse hepatitis virus), orthomyxovirus (influenza virus), rhabdovirus (vesicular stomatitis virus), herpes virus (cytomegalovirus), lentivirus (human immunodeficiency virus), and retrovirus (murine leukemia virus). After prolonged heating at 65 °C or heating for 90 sec at 103 °C, parvovirus (canine parvovirus) and the phage phiX174 were also completely inactivated. Papovavirus represented by simian virus 40 (SV-40) was the most heat-resistant virus evaluated. The infectivity of SV-40 was reduced by $10^4$ Tissue Culture Infectious Doses (TCID$_{50}$) per ml after 90 sec at 103 °C, but a marginal residual activity ($<1.5$ TCID$_{50}$ per ml) was observed. Subsequent pasteurization for 10 h at 65 °C did not further reduce the infectivity of SV-40. This study shows that the two heat-inactivation steps used during the production of this vaccine kill a wide variety of viruses that might be present in human blood.

Key words: hepatitis B vaccine, heat inactivation, viruses, tissue culture infectious doses (TCID$_{50}$)

INTRODUCTION

A plasma-derived hepatitis-B vaccine [Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB)], inactivated by two heating cycles (90 sec at 103 °C followed by 10 hr at 65 °C), is highly efficacious [Coutinho et al, 1983; Desmyter et al, 1983; Wong et al, 1984]. The safety of this vaccine has been established in chimpanzees [Reerink-Brongers et al, 1982], and a reduction of at least $10^{15}$ chimpanzee-infectious doses (CID$_{50}$) of HBV during manufacture of the vaccine has been observed.
[Lelie et al, 1987]. Since the introduction of this vaccine, over 20,000 human individuals have been immunized without important side effects.

The procedures used in the manufacture of another vaccine [Merck, Sharp and Dohme (MSD)] are effective in the inactivation of HBV [Tabor et al, 1983] and eight other virus groups [Gerety and Tabor, 1983]. The urea treatment inactivates rhabdovirus, poxvirus, togavirus, herpes virus, coronavirus, reovirus, myxovirus, and picornavirus. The pepsin treatment kills six of these virus groups but does not completely inactivate picornavirus as represented by mengovirus or myxovirus as represented by Newcastle disease virus. The effect of formalin has been reported to be rather modest in the inactivation of the human immunodeficiency virus (HIV) [Spire et al, 1984a] in contrast with the effect of heat in a liquid environment [Spire et al, 1984b; Martin et al, 1985; McDougal et al, 1985; Resnick et al, 1986]. Both formalin and heat inactivate agents of non-A/non-B hepatitis [Tabor and Gerety, 1980; Yoshizawa et al, 1982].

In this report, we describe the efficacy of the two heating steps applied during manufacture of hepatitis-B vaccine CLB in the inactivation of 12 virus families.

MATERIALS AND METHODS

Heat Inactivation of Viruses

90 sec at 103 °C. A human serum protein solution (ultracentrifugation resuspension) was prepared from plasma negative for HBsAg, anti-HBc and anti-HBs according to methods described for the purification of hepatitis-B vaccine [Lelie et al, 1987]. For the inactivation of canine parvovirus (CPV), phiX174, and simian virus 40 (SV-40), 1 to 5 ml of virus stock solution were added to 50 ml of this human serum solution containing 6 mg of protein per ml. This mixture was heated in a 100-ml closed bottle for 90 sec at 103 °C in an oil bath and then cooled in an ice bath. The solution was centrifuged for 1 hr at 48,000g and the clarified supernatant was sterile filtered. The temperature in the bottle was continuously monitored with a data-logging apparatus (Kaye Instruments). Samples taken from the unheated solution and after heat treatment were immediately frozen in an ethanol-CO₂ bath.

10 h at 65 °C. The above human protein solution was heated for 90 sec at 103 °C. The heated solution was clarified by centrifugation at 48,000g and was then sterile filtered. For the inactivation of vaccinia virus, sindbis virus, encephalomyocarditis virus (EMC), mouse hepatitis virus (MHV), influenza virus, vesicular stomatitis virus (VSV), cytomegalovirus (CMV), HIV, murine leukemia virus (MuLV), CPV, and phiX174, 1 to 5 ml of stock solution were dissolved in 50 ml of this protein solution containing 1 mg of protein per ml. This mixture was heated at 65 °C in a water bath, whereas in a dummy bottle filled with the same protein solution the temperature was continuously monitored. During the 15-min warming up and in the 10-hr pasteurization at 65 °C thereafter, several samples were taken and immediately frozen in an ethanol-CO₂ bath. In one virus-inactivation experiment, the solution of SV-40, heated for 90 sec at 103 °C, was used directly for subsequent pasteurization.

Infectivity Assays of Viruses

The infectivity titers of the sequential samples of the virus preparations before, during, and after heat inactivation were tested in 10-fold dilutions, in duplicate or quadruplicate. This was done by assessing the percentage of cell cultures at each dilution
TABLE I. Heat-Labile Viruses Completely Inactivated During a Period of 15 min Heating Up to 65 °C

| Virus                   | before warming up | after warming up |
|-------------------------|-------------------|------------------|
| Vaccinia (poxvirus)     | 10³⁸              | None             |
| EMC (picornavirus)      | 10⁹               | None             |
| Sindbis (togavirus)     | 10¹⁰              | None             |
| MHV (corona virus)      | 10⁷               | None             |
| Influenza (orthomyxovirus) | 10⁴          | None             |
| VSV (rhabdovirus)       | 10⁴               | None             |
| CMV (herpes virus)      | 10⁴               | None             |
| HIV (lentivirus)         | 10⁴               | None             |
| MuLV (retrovirus)       | 10⁴               | None             |

*Titers not known.

that showed cytopathic effect (CPE) or other markers of infection. The infectivity titers of
the inocula were expressed in Tissue Culture Infectious Doses (TCID₅₀), i.e., the highest
dilution in which 50% of the cell cultures still showed infection. EMC, sindbis virus, MHV
(kindly donated by Dr. B.A.M. van der Zeyst, Veterinary Faculty of the University of
Utrecht, The Netherlands), influenza virus (kindly given by Dr. B. van Steenis,
Rijksinstituut voor de Volksgezondheid en Milieu Hygiëne, Bilthoven, The Netherlands),
VSV, MuLV, phiX174, and HIV were tested at our laboratory according to standard
techniques. Vaccinia was obtained from and tested by Dr. B. van Steenis (RIV); Dr. J. van
der Noordaa (Academic Medical Centre, University of Amsterdam) kindly performed the
titrations of CMV and SV-40, and Dr. W.M. Hermann-Dekkers (Duphar B.V., Weesp,
The Netherlands) delivered CPV and tested the infectivity.

RESULTS

Table I summarizes the viruses that had already been inactivated within a period of
15 min that was required to reach a temperature of 65 °C. Although the infectivity titers of
the various preparations before heating varied considerably, no residual activity was
observed for nine virus families after the 15 min required to reach 65 °C.

Table II shows the heat-resistant viruses that were inactivated only after heating at
103 °C or prolonged heating at 65 °C. The infectivity of CPV was reduced by 10²³ TCID₅₀
per ml within 40 min at 65 °C and by at least 10⁵.⁵ TCID₅₀ per ml after both 10 hr at 65 °C
and 90 sec at 103 °C. The infectivity of phiX174 was already reduced by 10⁵.⁵ TCID₅₀ per
ml after 9 min of heating at 65 °C and no residual activity was observed after heating of
10⁷ TCID₅₀ per ml for 10 hr at 65 °C or for 90 sec at 103 °C. A reduction of 10⁴ TCID₅₀ per
ml of SV-40 was found after 90 sec of heating at 103 °C of 10⁵.⁵ TCID₅₀ per ml. The
marginal residual activity (≤10¹⁵ TCID₅₀ per ml) remained unaltered after subsequent
pasteurization for 10 hr at 65 °C. However, the protein concentration during the
pasteurization of the residual infectivity of SV-40 was 6-fold higher than the usual protein
concentration during pasteurization of the vaccine.

DISCUSSION

The experiments show that vaccinia virus, EMC, sindbis virus, MHV, influenza
virus, VSV, CMV, HIV, and MuLV are evidently highly sensitive to heat treatment,
TABLE II. Heat-Resistant Viruses Inactivated by Short Heating at 103 °C or by Prolonged Heating at 65 °C

| Virus      | Heat treatment                      | TCID$_{50}$ per ml before heating | TCID$_{50}$ per ml after heating |
|------------|-------------------------------------|-----------------------------------|----------------------------------|
| CPV (parvovirus) | 90 sec at 103 °C                   | $10^{5.5}$                        | None                              |
|            | 10 min at 65 °C                    | $10^7$                            | $10^{7.3}$                        |
|            | 40 min at 65 °C                    | $10^{6.9}$                        | $10^{7.4}$                        |
|            | 10 hr at 65 °C                     | $10^{5.3}$                        | None                              |
| phiX174 (bacteriophage) | 90 sec at 103 °C                  | $10^7$                            | None                              |
|            | 5 min at 65 °C                     | $10^{7.3}$                        | $10^{7.3}$                        |
|            | 9 min at 65 °C                     | $10^{7.0}$                        | $10^{1.4}$                        |
|            | 10 hr at 65 °C                     | $10^{7.0}$                        | None                              |
| SV-40 (papovavirus) | 90 sec at 103 °C                  | $10^{5.5}$                        | $10^{5.5}$                        |
|            | 90 sec at 103 °C, followed by 10 hr at 65 °C | $10^{5.7}$                        | $10^{5.7}$                        |

because no viral infectivity was recovered after the 15 min required to reach the temperature of 65 °C. At least $10^5$ TCID$_{50}$ per ml of CPV and $10^7$ TCID$_{50}$ per ml of the phage phiX174 were completely inactivated after prolonged pasteurization at 65 °C or after 90 sec heating at 103 °C. The infectivity of SV-40 was reduced by $10^4$ TCID$_{50}$ per ml after 90 sec at 103 °C, but a marginal residual infectivity of $\leq 10^1.5$ TCID$_{50}$ per ml was not eliminated by subsequent pasteurization. Because in this experiment the protein concentration during pasteurization was higher than during regular production of the vaccine, the inactivation of the final heating step for SV-40 may be underestimated as a consequence of too high a concentration of protective proteins. Similar results were reported by Rohwer [1983] who found heat-resistant subpopulations of phage $\lambda$ at 60 °C, but only in the presence of brain homogenate.

Our study confirms results of other investigators who have also established that HIV is effectively killed in a liquid environment [Spire et al, 1984; Martin et al, 1985; McDougal et al, 1985; Resnick et al, 1986]. A representative of the group of slow viruses was not included in our study, but Rohwer [1983] has reported that 97% of the heat-resistant scrapie agent was destroyed within 2 min of exposure to 100 °C in aqueous solution.

The absence of extraneous infectious agents in the starting plasma of each lot of the vaccine is confirmed according to the requirements of the World Health Organization [1980]. Hence, it is highly unlikely that batches of the vaccine that have passed this test contain residual infective virus after the purification and inactivation steps used.

ACKNOWLEDGMENTS

We thank Dr. J. van der Noordaa, Dr. B. van Steenis, and Dr. W.M. Hermann-Dekkers for their help with the determination of the viral infectivity titers, Dr. F. Miedema and Dr. M. Tersmette for the titrations of HIV. We also thank Dr. J. Moors, Mrs. L. Wilson, and Mr. E. de Jonge, who carried out the heat inactivation, and Mrs. P. Oehlers and Mrs. P. Treep-van Leeuwen, who carried out the virus titrations. Finally, we are indebted to Dr. B.Th. Visser and Dr. C. van Wisse for their critical comments.
REFERENCES

Coutinho RA, Lelie PN, Albrecht-van Lent P, Reerink-Brogers EE, Stoutjesdijk L, Dees PJ, Nivard J, Huismam JG, Reesink HW (1983): Efficacy of a heat-inactivated hepatitis-B vaccine in male homosexuals: Outcome of a placebo-controlled double-blind trial. British Medical Journal 286:1305–1308.

Desmyter J, Cobaert J, de Groote G, Reynders M, Reerink-Brogers EE, Lelie PN, Dees PJ, Reesink HW, the Leuven Renal Transplantation Collaborative Group (1983): Efficacy of heat-inactivated hepatitis-B vaccine in haemodialysis patients and staff: Double-blind, placebo-controlled trial. Lancet 2:1323–1327.

Gerety RJ, Tabor E (1983): Newly licensed hepatitis-B vaccine: Known safety and unknown risks. Journal of the American Medical Association 249:745–746.

Lelie PN, Reesink HW, Niessen J, Brotman B, Prince AM (1987): Safety of a plasma-derived hepatitis-B vaccine inactivated by two heating cycles: 90 seconds at 103 °C and 10 hours pasteurization at 65 °C. I. Inactivation studies of HBV in chimpanzees. Journal of Medical Virology 23:289–295.

Martin S, McDougal JS, Loskosi SI (1985): Disinfection and inactivation of the human T-lymphotropic virus type III: Lymphadenopathy-associated virus. Journal of Infectious Diseases 152:400–403.

McDougal JS, Martin LS, Cort SP, Mozen M, Helderbrant CM, Evatt BL (1985): Inactivation of the acquired immunodeficiency syndrome virus, human T-lymphotropic virus III/lymphadenopathy-associated virus, with special reference to antihemophilic factor. Journal of Clinical Investigation 76:875–877.

Reerink-Brogers EE, Reesink HW, Brummelhuis HGI, Schut BJTh, Dees PJ, Lelie PN, Raap AK, Wilson-de Stürler LA, van Aken WG, Balner H, van Eerd PMCA, van Schie ThC, Sitz LW, van Steenis B, Feltkamp-Vroom ThM (1982): Preparation and evaluation of heat-inactivated HbsAg as a vaccine against hepatitis B. In Szmuness W, Alter HJ, Maynard J (eds): “Viral Hepatitis.” Philadelphia: Franklin Institute Press, pp 437–449.

Resnick L, Veren K, Salahuddin SZ, Tondreau G, Markham PD (1986): Stability and inactivation of HTLV-III/LAV under clinical and laboratory environments. Journal of the American Medical Association 255:1887–1891.

Rohwer RG (1983): Virus-like sensitivity of the scrapie agent to heat inactivation. Science 223:600–602.

Spire B, Dormont D, Barré-Sinoussi F, Montagnier L, Chermmann JC (1984a): Inactivation of lymphadenopathy-associated virus by chemical disinfectants. Lancet 2:899–901.

Spire B, Barré-Sinoussi F, Montagnier L, Chermmann JC (1984b): Inactivation of lymphadenopathy-associated virus by heat, gamma rays and ultraviolet light. Lancet 1:188–189.

Tabor E, Gerety RJ (1980): Inactivation of an agent of human non-A, non-B hepatitis by formalin. Journal of Infectious Diseases 142:767–770.

Wong CW, Ip HMH, Reesink HW, Lelie PN, Reerink-Brogers EE, Yeung CY, Ma HK (1984): Prevention of the HBsAg carrier state in newborn infants of mothers who are chronic carriers of HBsAg and HBeAg by administration of hepatitis-B vaccine and hepatitis-B immunoglobulin. Lancet 1:921–926.

World Health Organization (1980): Proposed requirements for hepatitis-B vaccine. WHO Expert Committee on Biological Standardization, Geneva. WHO/BS/79.1239 pp 1–20.

Yoshizdwa H, Itoh Y, Iwarkiri S, Kitajima K, Tamaka A, Tachibana T, Nakamura T, Myakawa Y, Mayumi M (1982): Non-A, non-B (type 1) hepatitis agent capable of inducing tubular ultrastructures in the hepatocyte cytoplasm of chimpanzees: Inactivation by formalin and heat. Gastroenterology 82:502–506.