Minimum infectious dose of hepatitis B virus in chimpanzees and difference in the dynamics of viremia between genotype A and genotype C

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BACKGROUND: In planning optimal hepatitis B virus (HBV) blood screening strategies, the minimum infectious dose and early dynamics of HBV need to be determined for defining the window period for HBV DNA as well as for hepatitis B surface antigen (HBsAg).

STUDY DESIGN AND METHODS: Pairs of chimpanzees were inoculated with preacute-phase inocula containing HBV of genotype A or genotype C to determine the minimum infectious dose, and two pairs of chimps infected with the lowest infectious dose of genotypes A and C were followed for HBV markers.

RESULTS: The minimum 50 percent chimpanzee infectious dose (CID_{50}) was estimated to be approximately 10 copies for genotype A and for genotype C. In the two chimps inoculated with the lowest infectious dose, the HBV DNA window was 55 to 76 days for genotype A and 35 to 50 days for genotype C, respectively. The HBsAg window was 69 to 97 days for genotype A and 50 to 64 days for genotype C, respectively. The doubling times of HBV DNA were 3.4 days (95% confidence interval [CI], 2.6-4.9 days) for genotype A and 1.9 days (95% CI, 1.6-2.3 days) for genotype C. When comparing the replication velocity of HBV DNA between the two genotypes, the doubling time of genotype C was significantly shorter than that of HBV genotype A ($p < 0.01$).

CONCLUSION: Although the CID_{50} of approximately 10 copies was similar for the two HBV genotypes, the doubling time and pre-HBV nucleic acid amplification technology (<100 copies/mL) window period in chimps infected with the lowest infectious dose seemed to be shorter for genotype C than for genotype A.

Posttransfusion infection with hepatitis B virus (HBV) has decreased dramatically since screening for hepatitis B surface antigen (HBsAg) was introduced in the early 1970s. The number of reported posttransfusion hepatitis B cases has been further reduced after screening for antibody to HBV core (anti-HBc) was implemented in the late 1980s in the United States and Japan.[1,2] Japan introduced HBV DNA screening by nucleic acid amplification technology (NAT) in minipools (MPs) in 1999. Since introduction of MP-NAT, more than 500 seronegative donations with detectable HBV DNA have been interdicted, although there are still units of blood in an early or late phase of HBV infection

ABBREVIATIONS: CID_{50} = 50 percent chimpanzee infectious dose; CLIA = chemiluminescent immunoassay; JRC = Japanese Red Cross; MP(s) = minipool(s).

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with low viral load that can escape detection by NAT.[3] Interestingly, the lookback program of the Japanese Red Cross (JRC) demonstrated that low viral load donations in the window phase were more than 10-fold more often implicated in HBV transmission reports than were occult carriers with anti-HBc titers below the exclusion limit of the anti-HBc hemagglutination inhibition screening assay.[3] Bearing in mind the relatively high infectivity of HBV in the window phase, exact knowledge on early dynamics of HBV replication is important for residual risk estimations.[4-7] It will determine the threshold of NAT in identifying blood donors during the preacute phase of HBV infection, which is important for planning and executing evidence-based hepatitis B blood screening strategies.[7,8] In this context, the relative infectivity of HBV in the early window phase is an important factor for measuring the effect of NAT screening systems on the residual risk of HBV transmission by blood transfusion.[7-9]

Chimpanzees are the only experimental animals susceptible to human hepatitis viruses and have been very useful in transmission studies.[10] As early as the mid-1970s, it was demonstrated that blood units from HBV carriers, especially those positive for the presence of hepatitis B e antigen (HBeAg), have a tremendously high infectious potential and can transmit infection to chimps by intravenous inoculation with 1 mL of plasma diluted to $1:10^8$.[11] Now that NAT enables detection of HBV DNA in blood donors even in individual-donation format, it can be estimated by mathematical modeling what the residual risk would be depending on the minimum copy number required for infection.[9] More conservative risk modeling assumes that a single copy of HBV, if it successfully reaches a hepatocyte in susceptible hosts, may be enough to establish infection.[5] To pursue strategies for preventing HBV infections by blood transfusions, additional information on the infectivity of HBV is crucially required. It is imperative to define not only the minimum copy number of HBV DNA or number of virions required for transmission of HBV, but also the early dynamics of HBV replication in the circulation of infected hosts. This can be established more accurately in chimpanzee experiments. In this report on experimental transmission of hepatitis B in chimps, the minimum infectious dose was determined separately for HBV of genotypes A and C, and the copy number of HBV DNA for establishing infection was defined for each of them. Moreover, the doubling time and logarithmic time of HBV DNA were determined by following the viral dynamics in the preacute phase of chimpanzees who had received the minimum infectious dose of HBV.

MATERIALS AND METHODS

Chimpanzees

Six chimps entered this study. Their age, sex, and weight, as well as the HBV inocula that they received are listed in Table 1 along with the infection outcome. Every chimp was kept in an individual cage and received humane care in accordance with all relevant requirements for the use of primates in an approved institution. None of the six chimps had serologic or molecular biologic evidence of past or present HBV infection prior to the inoculation. They were also not infected with hepatitis C virus (HCV) and human immunodeficiency virus type-1. Chimps were inoculated intravenously while they were under anesthesia by intramuscular injection with ketamine hydrochloride. After the inoculation, serum samples were obtained once a week or more frequently as required, until 16 weeks or longer. They were tested for HBV DNA, HBsAg, anti-HBc, anti-HBs, and alanine aminotransferase as well as aspartate aminotransferase.

Inocula containing HBV

The chimpanzees received four kinds of inocula (Table 1). Inocula I and III were fresh-frozen plasma (FFP) units from blood donors acutely infected with HBV genotypes A and C, respectively. Inocula II and IV were plasma samples from chimps infected with inoculum I of genotype A and inoculum III of genotype C, respectively (see Figs. 1A and 1B). Inocula II and IV were: 1) recovered in the preacute phase of HBV infection before immune responses of the host had developed; 2) positive for the presence of HBV DNA in the highest titer before anti-HBc developed; and 3) taken with utmost care to maintain the infectious activity and avoid attenuation during serial processing from blood collection until storage. Immediately after blood drawing

TABLE 1. Six chimpanzees and HBV inocula and HBV infection outcomes

Chimpanzee	Age, sex, weight	HBV DNA copies	Outcome
Inoculum I: FFP from a human donor in the preacute phase of HBV infection of genotype A			
1 Chimp 246	13 years, male, 60.7 kg	1 mL (6.9×10^4 copies/mL)	Infected
Inoculum II: Preacute-phase plasma of Chimp 246 containing HBV (2.6×10^6 copies/mL)			
2 Chimp 272	9 years, male, 58.7 kg	1 mL ($1:10^6$ dilution)	Not infected
3 Chimp 279	8 years, male, 51.4 kg	1 mL ($1:10^6$ dilution)	Not infected
3 Chimp 279	Reinoculation	1 mL ($1:10^5$ dilution)	Infected
4 Chimp 280	8 years, male, 39.4 kg	1 mL ($1:10^5$ dilution)	Infected
Inoculum III: FFP from a human donor in the preacute phase of HBV infection of genotype C			
2 Chimp 272	Reinoculation	5 mL (5.3×10^5 copies/mL)	Infected
Inoculum IV: Preacute-phase plasma of Chimp-272 containing HBV (3.0×10^6 copies/mL)			
5 Chimp 269	11 years, male, 62.5 kg	1 mL ($1:10^6$ dilution)	Not infected
6 Chimp 285	7 years, male, 41.1 kg	1 mL ($1:10^6$ dilution)	Not infected
5 Chimp 269	Reinoculation	1 mL ($1:10^5$ dilution)	Infected
6 Chimp 285	Reinoculation	1 mL ($1:10^5$ dilution)	Infected

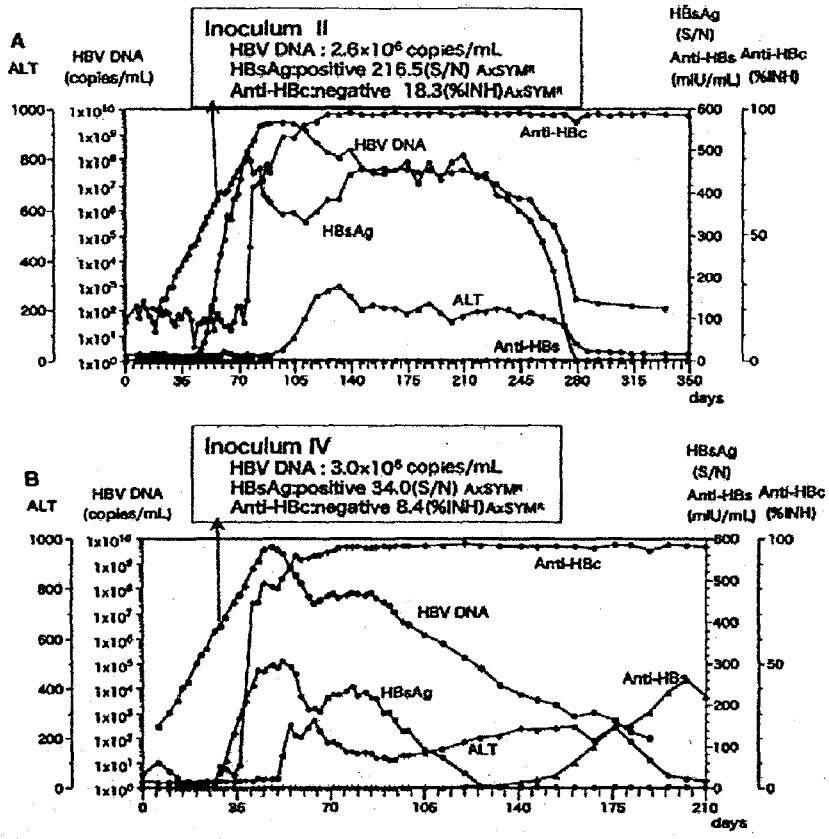

Fig. 1. Time course of HBV serum markers in chimps used as a source of inoculation of HBV genotype A and genotype C. (A) Chimp 246 was inoculated with human plasma of HBV genotype A. Inoculum II for chimp infectivity studies was harvested in the ramp-up phase of viremia before anti-HBc seroconversion at the time HBV DNA had reached a concentration of 2.6×10^6 copies per mL and the HBsAg response had increased to a signal-to-noise (S/N) ratio of 216.5 (cutoff S/N = 2.0). (B) Chimp 272 was inoculated with human plasma of HBV genotype C. Inoculum IV was harvested in the ramp-up phase of viremia before anti-HBc seroconversion at the time HBV DNA had reached a concentration of 3.0×10^6 copies per mL and HBsAg had increased to an S/N ratio of 34.0.

from chimps, plasma samples were separated. They were divided into 15 tubes in 1-mL aliquots, snap-frozen in liquid nitrogen, and kept in a deep freezer at –80°C until used for transmission experiments. For each experiment, the plasma in one tube was thawed gently by immersing it in a water bath at 37°C, and the required amounts were used.

Laboratory tests

HBsAg, anti-HBc, and antibody to HBsAg (anti-HBs) were determined by chemiluminescent immunoassay (CLIA) with commercially available kits (AxSYM, Abbott Japan, KK, Tokyo, Japan), with the index of 2.0 (signal-to-noise [S/N]), 50 percent inhibition, and 5.0 mIU per mL as cutoff values, respectively. Qualitative assay for HBV DNA was performed by polymerase chain reaction (PCR) with primers deduced from the S region of HBV DNA.[12] HBV DNA was quantitated by the PCR (TaqMan, Roche Diagnostics KK, Tokyo, Japan) with a sensitivity of 100 copies per mL. Quantitative assays for HBV DNA were performed simultaneously for an accurate comparison of data.

Calculation for doubling time and logarithmic time of HBV DNA

To calculate the doubling time and the logarithmic time (time for reaching 10 times the amount) of HBV DNA at ramp-up after the infection, HBV DNA copy numbers were evaluated statistically by log linear regression analysis. The comparison of regression slope (the doubling time and the logarithmic time) between HBV genotypes was evaluated by growth curve analysis (Vonesh-Carter-Ohtaki method).[13,14]

RESULTS

Inocula and copy numbers of HBV genotype A or genotype C recovered from chimpanzees in the preacute phase of infection

Chimp 246 was injected intravenously with 1 mL of FFP from a blood donor in the preacute phase of HBV infection (Table 1); the donor had been screened by NAT at a JRC Blood Center. His plasma sample contained 6.9×10^4 copies per mL of HBV DNA genotype A and was positive for the presence of HBsAg but negative for the presence of anti-HBc (inoculum I). Plasma was harvested from Chimp 246, in the preacute phase of HBV infection 57 days after inoculation (inoculum II). It contained 2.6×10^6 copies per mL HBV DNA and was positive for the presence of HBsAg but still negative for the presence of anti-HBc and anti-HBs (Fig. 1A).

Likewise, Chimp 272 was injected intravenously with 5 mL of FFP from a blood donor in the preacute phase of HBV infection who had been screened by NAT at JRC (Table 1). It contained 5.3×10^5 copies per mL of HBV DNA genotype C and was positive for the presence of HBsAg but negative for the presence of anti-HBc and anti-HBs (inoculum III). Thus, Chimp 272 was inoculated with

2.7×10^6 copies of HBV genotype C. The preacute plasma sample was collected from Chimp 272 29 days after challenge (inoculum IV). It contained 3.0×10^6 copies per mL HBV DNA and was positive for the presence of HBsAg but still negative for the presence of anti-HBc and anti-HBs (Fig. 1B).

Estimates of HBV DNA copy numbers in serial 1-in-10-fold dilutions and inocula below the HBV NAT detection limit

Serial 1-in-10 dilutions of inoculum II of genotype A were prepared in preinoculation serum sample from each chimp (e.g., Chimp 272, Chimp 279, and Chimp 280, respectively). Dilutions were delivered to three tubes each in 1-mL aliquots and snap-frozen in liquid nitrogen. Concentration of HBV DNA was determined in one of the three tubes in each dilution so as to guarantee copy numbers of HBV DNA in the other two vials that were inoculated into chimps. These samples had been stored in a deep freezer at –80°C until inoculation.

Table 2 shows the measured HBV DNA concentrations in 1-in-10 dilutions of inoculum II (genotype A). The quantitative HBV DNA results starting from 2.6×10^6 copies per mL in the undiluted sample varied between 2.0×10^5 to 2.3×10^5, 2.0×10^4 to 2.4×10^4, 1.6×10^3 to 2.0×10^3, and 1.7×10^2 to 2.8×10^2 copies per mL, respectively, in the 1:10, $1:10^2$, $1:10^3$, and $1:10^4$ dilutions. These quantitative results are an indication of the accuracy of the dilution and assay procedure. On the premise that dilutions beyond $1:10^4$ had been performed properly, further dilutions to $1:10^5$ and $1:10^6$ would have contained 16 to 28 and 1.6 to 2.8 HBV DNA copies per mL (ranges estimated by variations of HBV DNA measurements in lower dilutions), respectively, although they were below the detection limit of the PCR method used.

Likewise, serial 1-in-10 dilutions of inoculum IV (genotype C) were prepared in the plasma sample from Chimp 269 and Chimp 285. HBV DNA in 3.0×10^6, 3.5×10^5 to 3.8×10^5, 3.6×10^4 to 3.9×10^4, 3.6×10^3 to 4.6×10^3, and 4.3×10^2 to 4.6×10^2 copies per mL were detected in the original serum samples at 1:10, $1:10^2$, $1:10^3$, and $1:10^4$ dilutions thereof, respectively (Table 3). Thus, further experiments were performed on the assumption that serial dilutions of $1:10^5$ and $1:10^6$ of inoculum IV would have contained 35 to 46 and 3.5 to 4.6 HBV DNA copies per mL, respectively.

Determination of the minimum copy number required for transmission of HBV genotype A or genotype C to chimpanzees

When Chimp 272 and Chimp 279 were inoculated intravenously with 1.0 mL of inoculum II diluted $1:10^6$ (equivalent to 1.6 to 2.8 copies of HBV DNA in an in vitro assay), HBV infection did not develop in either of them during monitoring for 119 days (17 weeks) and thereafter. Chimp 279 was then rechallenged with 1.0 mL of inoculum II diluted $1:10^5$ (equivalent to 16-28 copies). He then became infected and developed HBV DNA in his serum 55 days (8 weeks) after the inoculation. Chimp 280 was also inoculated intravenously with 1.0 mL of inoculum II diluted $1:10^5$ (equivalent to 16 to 28 copies of HBV DNA). He developed HBV DNA in the circulation 76 days (11 weeks) after infection. In view of the incubation period of 55 to 76 days (8-11 weeks) for $1:10^5$ dilution of inoculum II, HBV infection would probably not have occurred in chimps who received $1:10^6$ dilution if they had been followed longer than 119 days (17 weeks).

Chimp 269 and Chimp 285 were inoculated with 1.0 mL of inoculum IV diluted $1:10^6$ (equivalent to 3.5-4.6 copies of HBV DNA in an in vitro assay). During follow-up

TABLE 2. Quantification of HBV DNA in serial 1-in-10 dilutions of the standard serum for HBV genotype A (inoculum II)*

		Serial dilutions in preinoculation serum samples of each chimpanzee					
Chimpanzee	Undiluted	1:10	$1:10^2$	$1:10^3$	$1:10^4$	$1:10^5$	$1:10^6$
272	2.6×10^6	2.3×10^5	2.0×10^4	2.0×10^3	1.7×10^2	Not done	<100
279	2.6×10^6	2.0×10^5	2.4×10^4	2.0×10^3	2.4×10^2	<100	<100
280	2.6×10^6	2.3×10^5	2.3×10^4	1.6×10^3	2.8×10^2	<100	Not done

* Data are reported as copies per mL.

TABLE 3. Quantification of HBV DNA in serial 1-in-10 dilutions of the standard serum for HBV genotype C (inoculum IV)*

		Serial dilutions in preinoculation serum of each chimpanzee					
Chimpanzees	Undiluted	1:10	$1:10^2$	$1:10^3$	$1:10^4$	$1:10^5$	$1:10^6$
Chimp 269	3.0×10^6	3.8×10^5	3.9×10^4	3.6×10^3	4.6×10^2	<100	<100
Chimp 285	3.0×10^6	3.5×10^5	3.6×10^4	4.6×10^3	4.3×10^2	<100	<100

* Data are reported as copies per mL.

for 112 days (16 weeks), however, no HBV infection occurred in either of them. Subsequently, they were rechallenged with 1.0 mL of inoculum IV diluted $1:10^5$ (equivalent to 35-46 copies of HBV DNA) 17 weeks after the initial inoculation. They developed HBV DNA in the circulation 35 and 50 days thereafter, respectively, indicating that both of them were infected. Therefore, the 50 percent chimp infectious dose (CID_{50}) for both genotype A and genotype C lies between the lowest infectious dose of approximately 30 copies and the subinfectious dose of approximately 3 copies or at approximately 10 HBV DNA copies.

HBV infection resolved in all six chimps and they never became carriers. Within a few weeks after the peak HBV DNA titer was reached, serum levels of transaminase increased slightly, within 3 times the upper limit of normal.

Replication velocity of HBV DNA in the preacute phase of infection

Doubling time and logarithmic time of HBV genotype A

Figure 2A illustrates the appearance of HBV genotype A in the circulation, when HBV DNA reached more than 10^2 copies per mL, as well as its early dynamics in Chimp 246, Chimp 279, and Chimp 280 during the preacute phase of exponential replication. HBV DNA emerged in the circulation earlier in Chimp 246 than the other two chimps, but

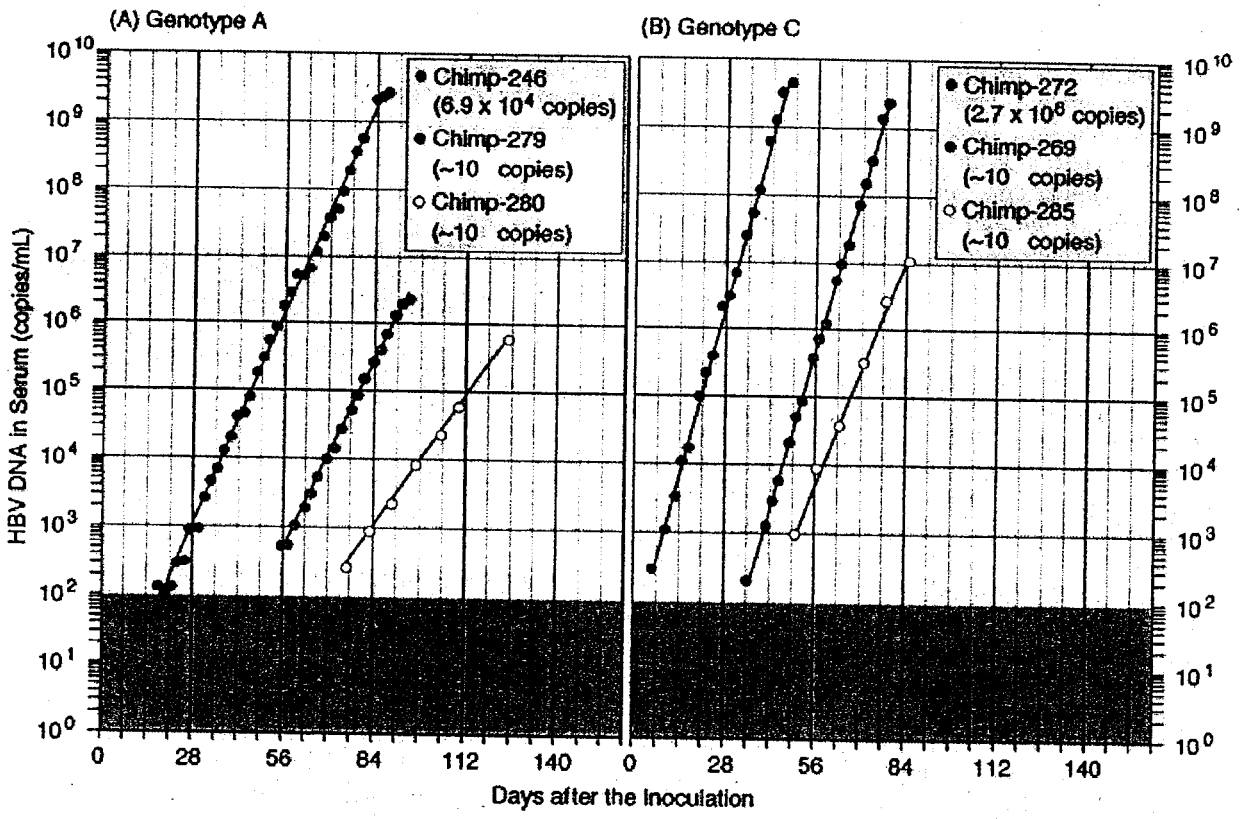

Fig. 2. Log-linear increase of HBV DNA in the circulation of chimpanzees during the early exponential replication phase. (A) Dynamics in the early ramp-up phase of viral DNA for three chimps inoculated with HBV genotype A: one chimp (Chimp 246) received 1 mL of human plasma containing 6.9×10^4 copies and the other two chimps (Chimps 279 and 280) received 1 mL of a 100,000 dilution of chimp plasma taken in the HBsAg ramp-up phase just before appearance of anti-HBc, which dilution contains a measured amount of 16 to 28 copies. (B) Graph summarizes the viral load dynamics for three chimpanzees inoculated with HBV genotype C: one chimp (Chimp 272) received 5 mL of human plasma with 2.7×10^6 copies of HBV DNA and the two other chimps (Chimps 269 and 285) received a measured amount of 35 to 46 copies (1:100,000 dilution) of preacute-phase chimpanzee plasma. Shaded areas are below the detection limit of NAT (<100 copies/mL). Only the phase of exponential replication is shown, and HBV DNA decreased after it reached peak values of 5.7×10^5 to 2.8×10^9 copies per mL in three chimps inoculated with HBV genotype A and 1.1×10^7 to 4.6×10^9 copies per mL in three chimps inoculated with HBV genotype C.

this animal had received more than a 1000-fold larger amount of copies of HBV than the other two chimps. Despite the 1000-fold higher infectious dose, the log-linear increase of HBV DNA in Chimp 246 was the same as in Chimp 279, who had received the minimum infectious dose. In Chimp 246, HBV DNA replicated exponentially from 21 to 97 days (3-13 weeks) until it peaked and then declined. Even though the same minimum infectious dose of HBV was inoculated, Chimp 279 developed detectable HBV DNA about 21 days (3 weeks) earlier than Chimp 280, in whom HBV replicated slightly slower. Despite differences in HBV doses and individual variation, the replication velocity was constant for HBV genotype A in the preacute phase of infection, before innate immune responses of the host developed, while the virus replicated at an exponential rate. The doubling time and the logarithmic time, in the early exponential viral replication phase, were calculated to be 2.7 to 4.4 and 9.0 to 14.7 days, respectively (see Table 4).

TABLE 4. Estimated doubling times and logarithmic times for HBV genotypes A and C with log-linear and growth-curve analysis

Genotype	Doubling time (days)	Logarithmic time (days)	$y = a \times \exp(b \times x)$ a	b	R^2
Genotype A					
Chimp 246	2.71	9.01	0.8491	0.2556	0.997
Chimp 279	3.05	10.14	0.0015	0.2271	0.998
Chimp 280	4.43	14.73	0.0022	0.1563	0.999
Genotype C					
Chimp 272	1.68	5.58	0.2074	0.413	0.998
Chimp 269	1.79	5.96	0.0002	0.3863	0.999
Chimp 285	2.5	8.31	0.0009	0.2771	0.997

TABLE 5. Comparing the replication velocity of HBV DNA between the two genotypes estimated by a growth curve

Genotype	Doubling time (95% CI), days	Logarithmic time (95% CI), days	$y = a \times \exp(b \times x)$ a*	b (95% CI)	p Value
A	3.44 (2.64-4.89)	11.42 (8.80-16.26)	2.299	0.2017 (0.14-0.26)	<0.01
C	1.9 (1.63-2.27)	6.3 (5.41-7.54)	2.299	0.3654 (0.31-0.43)	

* To evaluate the difference of "b" (that is, slope) between the two genotypes, the growth curve model is assuming that "a" is identical.[13]

Doubling time and logarithmic time of HBV genotype C

The replication velocity in the preacute phase of infection in chimpanzees inoculated with genotype C inocula was faster than in the chimps infected with HBV of genotype A (Fig. 2B). Again, slight variation in log-linear increase of HBV DNA was found, and HBV DNA appeared in serum earlier in Chimp 272 who was inoculated with a 100,000-fold higher infectious dose than was administered to Chimps 269 and 285. As seen in the chimps inoculated with HBV genotype A, HBV genotype C increased in a log-linear fashion in the absence of host immune responses. Doubling times of HBV DNA in the circulation of Chimp 272, Chimp 269, and Chimp 285 were calculated to be 1.7 to 2.5 days and logarithmic times were 5.6 to 8.3 days as determined with the regression formula shown in Table 4.

When comparing the replication velocity of HBV DNA between the two genotypes estimated by a growth curve, the difference was significant ($p < 0.01$, Table 5). That is, the doubling time of replications of HBV DNA with genotype A was estimated to be 3.44 days (95% confidence interval [CI], 2.64-4.89 days) and the logarithmic time was estimated to be 11.42 days (95% CI, 8.80-16.26 days). By contrast, those with HBV genotype C were estimated to be 1.90 days (95% CI, 1.63-2.27 days) and 6.30 days (95% CI, 5.41-7.54 days), respectively.

TABLE 6. Window periods before HBV DNA and HBsAg developed in the circulation of chimpanzees inoculated with the minimum infectious dose of genotype A or genotype C

HBV inoculated	Chimp infected	Markers of HBV infection: HBV DNA (days)	HBsAg (days)
Genotype A	279	55	69
	280	76	97
Genotype C	269	35	50
	285	50	64

Window periods of HBV DNA and HBsAg in chimpanzees inoculated with the minimum infectious dose of HBV

After inoculation, the time before HBV DNA becomes detectable in the circulation by the single-sample NAT (with a sensitivity of 10^2 copies/mL) and the time before HBsAg was detected by CLIA after inoculation are listed in Table 6. The HBV DNA (<100 copies/mL) NAT window was 55 and 76 days, respectively, in Chimp 279 and Chimp 280 inoculated with the lowest infectious dose of HBV genotype A (approx. 30 copies). These NAT window periods were longer than the 35 and 50 days, respectively, found in Chimp 269 and Chimp 285 inoculated with the lowest infectious amounts of HBV genotype C (approx. 30 copies). Likewise, the HBsAg window was longer in Chimp 279 and Chimp 280 infected with genotype A than in Chimp 269 and Chimp 285 infected with genotype C (69 and 97 days, respectively, vs. 50 and 64 days, respectively).

DISCUSSION

Animal models sensitive to human hepatitis viruses offer robust advantages in obtaining basic data of viral infectivity.[10] By experimental infection of chimps with HCV, we have been able to determine the minimum infectious dose of HCV required for establishing infection.[15,16] The doubling time of HCV was determined to be 6.3 to 8.6 hours in two chimps inoculated with the minimum infectious dose of approximately 10 copies of HCV RNA. During the first 5 days after inoculation, HCV RNA did not increase above the NAT detection limit of 10^2 copies per mL in the circulation.[16] It would not be possible to detect HCV infection during the initial few days after exposure, even if 1-mL samples were used for individual NAT.

In this study, we have determined the minimum infectious dose for two standardized inocula containing defined copy numbers of HBV DNA. They were plasma passages of HBV in chimps harvested during the preacute phase of infection and had been processed with the utmost care for maintaining infectious activity. The minimum infectious dose of HBV or the dose where 50 percent of the chimps would be infected lies between 1-in-1 million and 1-in-100,000 dilution of the original inocula and is estimated to be of the order of 10 copies, as was the case for HCV.[15] On the basis of HBV DNA concentrations measured in serial dilutions of inocula (Tables 2 and 3), the minimum infectious dose can be determined to be 16 to 28 copies for HBV genotype A and 35 to 46 copies for HBV genotype C.

There are two definitions of the minimum infectious dose of HBV. Theoretically, it is a single copy of HBV. Not all HBV virions entering the circulation of recipients, however, will succeed in reaching hepatocytes, because some of them are phagocytized by circulating macrophages and Kupffer cells in the sinusoids of the liver. In a mathematical window-phase risk model, Weusten and colleagues[9] have proposed a minimum infectious dose approximately 10 copies of HBV, on the basis of the CID_{50}.[17-19] Recently the inocula derived from chronic HBV carriers used in older chimpanzee studies[17,18] were requantified by Hsia and coworkers[20] with real-time TaqMan PCR. The estimated HBV copy number per CID_{50} (geq) was 169 for genotype A *adw*, 78 for genotype D *ayw*, and 3 for genotype C *adr*, calculated by mathematical division, respectively. These viral load data, performed on cryopreserved aliquots from an inocula derived from a chronic HBV carrier (i.e., HBsAg- and anti-HBc–positive), were derived retrospectively several decades after the chimp titration studies. These results are different from the results obtained in our study, where the inocula was derived from the early ramp-up phase of viremia (HBsAg is positive but anti-HBc is negative) and the chimp titration and viral load analyses were performed prospectively. Hence, the minimum infectious dose defined as a single copy, proposed on a theoretical basis, would deserve revisiting in practical HBV infections. The window period of HBV infection changes with the size of the inoculum. The more copies of HBV inoculated therefore the shorter the incubation period in experimental transmission studies in chimps.[11] An inverse correlation is reported, also, between time before HBsAg appears in serum and the HBV dose in human beings.[21] In accordance with these reports, we also found that the NAT window was shorter in chimps receiving larger sizes of inocula both for genotypes A and C (Fig. 2). The NAT (<100 copies/mL) window period was approximately 1 week with an inoculum of 2.7×10^6 copies of genotype C, approximately 3 weeks with 6.9×10^4 copies of genotype A, 5 to 7 weeks when inoculating 35 to 46 copies of genotype C, and 8 to 11 weeks when inoculating 16 to 28 copies of HBV genotype A, while no infection was observed during 16 to 17 weeks of observation with an inocula of approximately 3 copies of genotype A or B. Theoretically, HBV infection might have become detectable after 17 weeks, but this is unlikely when extrapolating the data above. Inoculation with HBV in large amounts, as happens with transfusion with HBsAg-positive blood units, has been largely excluded since introduction of HBsAg testing in 1972. Barker and Murray[21] have shown that inoculation of lower infectious doses of HBV in the range of 10^4 to 10^7 diluted icteric plasma no longer caused clinical hepatitis in healthy individuals, while infection still occurred with up to a 10^7 diluted inoculum, as detected by an HBsAg complement fixation test. Our study showed that HBV DNA levels increase 6.5×10^3 to 2.2×10^5 copies per mL at the time of the first HBsAg-reactive sample in six chimpanzees in whom blood samples were taken at intervals of 2 to 7 days. These amounts are enough to cause clinical hepatitis B.[21] Indeed, Satake and coworkers[3] found that transmission of 5,000 to 50,000 copies of HBV by blood components with a low viral load in the pre-MP-NAT window phase could cause clinical hepatitis B. Transfusion-transmitted HBV after introduction of individual-donation or small-pool NAT (<10) is still possible, but would involve relatively low infectious doses of HBV of approximately 10 to 100 CID_{50}.

In the chimps inoculated with approximately 30 copies of HBV, the NAT window was determined by individual-donation NAT having a sensitivity of 10^2 copies per mL, while the HBsAg window was established by CLIA with the highest sensitivity presently available.[5,12] The NAT window was 55 to 76 days and HBsAg window was 69 to 97 days, respectively, in Chimp 279 and Chimp 280 who had been inoculated with approximately 30 copies of HBV genotype A. In contrast, the NAT window was 35 to 50 days and the HBsAg window was 50 to 64 days, respectively, for Chimp 269 and Chimp 285 inoculated with approximately 30 copies of HBV genotype C. Thus, neither

the NAT nor the HBsAg window phases overlapped between minimum-dose infections of HBV genotypes A and C; they were longer for genotype A than genotype C. It may be that the NAT window is longer for genotype A, prevalent in Western countries, than genotype C common in Japan. It cannot be excluded, however, that the results observed in our inoculation studies with a limited number of chimpanzees were influenced by the host rather than the genotype of the virus. The duration of the NAT and HBsAg windows are influenced at least by three factors: 1) the infectious dose, 2) individual variation among recipients, and 3) distinct HBV genotypes.

We found the replication velocity of HBV in the preacute phase of infection remarkably different between genotypes A and C. From three chimps infected with HBV genotype A, the doubling time was estimated to be 3.44 days (95% CI, 2.64-4.89 days) and the logarithmic time 11.42 days (95% CI, 8.80-16.26 days). From three chimps infected with HBV genotype C, the doubling time was estimated to be 1.90 days (95% CI, 1.63-2.27 days), and the logarithmic time 6.30 days (95% CI, 5.41-7.54 days). Also in chimeric mice with the liver replaced by human hepatocytes, genotype A was found to replicate much slower than genotype C in the initial weeks of HBV infection.[22]

The replication velocity of HBV in the circulation, indicated by the viral doubling time, is an important factor when calculating the window-period reduction provided by NAT screening systems. Biswas and colleagues[5] calculated a doubling time of 2.56 days (95% CI, 2.24-2.97 days) based on a seroconversion panel of 23 HBV infections. Yoshikawa et al.[4] followed 93 donors in preacute phase HBV infections who had been identified by the routine NAT screening program on 50-MPs at JRC Blood Centers. They estimated a median doubling time of HBV at 2.6 days (range: 1.3-15.2).

Kleinman and Busch[7] have assessed the HBsAg window period based on the HBV doubling time of 2.56 days documented by Biswas and colleagues.[5] They estimated an HBsAg window at 38.3 days (95% CI, 33.0-43.7 days) by the CLIA HBsAg seroconversion point at a concentration of 1650 copies per mL, while Minegishi and coworkers[12] determined the HBsAg seroconversion point at 2100 copies per mL. We found the HBsAg seroconversion with AxSYM occurred when the HBV DNA concentration reached a level of 6.5×10^3 to 2.2×10^5 in six chimpanzees. The differences in HBV levels at HBsAg seroconversion in CLIA may be related to the genotype, but also could reflect differences in the calibration of HBV quantitative assays in genome copies.

It is not known if the chimpanzee model is as susceptible for HBV infection as human beings. As a result, the minimum dose of HBV for transmitting infection to man is, in fact, not precisely known. Nevertheless, a minimum human infectious dose of approximately 10 HBV DNA copies, as indicated by our chimpanzee infectivity experiments, seems a reasonable assumption for modeling the HBV transmission risk in the pre-HBV-NAT window period.

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識別番号・報告回数		回	報告日 年　月　日	第一報入手日 2008 年 2 月 8 日	新医薬品等の区分 該当なし	総合機構処理欄
一般的名称			研究報告の公表状況	An international collaborative study to establish the 2nd World Health Organization International Standard for hepatitis B virus DNA nucleic acid amplification technology-based assays. Baylis, S. A. et al, Vox Sanguinis (2008) [Epub ahead of print]	公表国 英国	
販売名（企業名）						
研究報告の概要	1999 年, WHO は核酸増幅検査 (NAT) による B 型肝炎ウイルス (HBV) DNA 検出のための最初の国際標準用品（サンプル 1）を樹立し, 10E6 IU/mL の力価を適用した。その当時, 将来的に代替標準品となる可能性があるという発想から, 同じ血漿から同じ凍結乾燥条件下で調製した別の DNA 検体（サンプル 2）が保存された。本試験の目的は, 長期間保存したこれらサンプルの力価及び安定性を再評価することであった。サンプル 1 及び 2 は, 6 ヵ所の分析機関に送付され, 4 回に分けて質と量の両面から分析した。全ての分析機関で得られた結果から, これら検体の力価に有意な差は認められなかった。また, 安定性試験では両検体ともに非常に安定しており, 4℃又は 20℃で 51 ヵ月以上保存した後でも有意な変性は認められなかった。この結果を受けて, WHO は 2006 年 10 月, サンプル 2 を NAT 法による HBV DNA 検出のための新たな国際標準品として樹立した。					使用上の注意記載状況・その他参考事項等 BYL-2008-0308

報告企業の意見	今後の対応
この試験は新たな国際標準品の樹立を報告するとともに, 凍結乾燥により保存された HBV DNA が極めて安定であることが報告されている。	現時点で新たな安全対策上の措置を講じる必要はないと考える。

Vox Sanguinis (2008)

REPORT

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An international collaborative study to establish the 2nd World Health Organization International Standard for hepatitis B virus DNA nucleic acid amplification technology-based assays

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Vox Sanguinis

Background and Objectives The aim of this study was to replace the 1st World Health Organization International Standard for hepatitis B virus DNA for nucleic acid amplification technique (NAT)-based assays (code 97/746) with a new International Standard. Two lyophilized preparations freeze dried from the same bulk were evaluated in the original collaborative study (coded 97/746 and 97/750, and termed AA and BB, respectively, in the original study). This present study re-evaluates these two preparations in terms of potency and real-time stability.

Materials and Methods The 1st International Standard (97/746) and the second lyophilized preparation (97/750) were coded Samples 1 and 2, respectively, in the present study. The samples were distributed to six laboratories and assayed on four separate occasions. Accelerated thermal degradation samples of the two preparations were examined after long-term storage at 4 °C and 20 °C for more than 51 months.

Results Data were returned from a total of nine different NAT-based assays, five in qualitative format and four in quantitative format. The results of this study confirm the results of the original collaborative study, with no significant differences being found in estimated international units (IU)/ml or polymerase chain reaction-detectable units/ml for the 1st International Standard (Sample 1 in this study) and the proposed replacement preparation, Sample 2 (97/750). Real-time and accelerated degradation studies indicate that both samples are very stable. Storage of both preparations at 20 °C for more than 51 months resulted in no detectable degradation.

Conclusions On the basis of the data presented in this collaborative study, Sample 2 (code 97/750) was established as the 2nd International Standard for hepatitis B virus DNA for NAT-based assays with a potency of 10^6 IU/ml (500 000 IU/vial).

Key words: hepatitis B virus, International Standard, NAT.

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Introduction

The 1st International Standard (IS) for hepatitis B virus (HBV) DNA for nucleic acid amplification technique (NAT)-based assays (code 97/746) was established in 1999 by the

World Health Organization (WHO) Expert Committee on Biological Standardization (ECBS) [1]. This standard has been used in the calibration of secondary standards and working reagents, and has been used in the validation of assays for both the qualitative and quantitative detection of HBV DNA in serum and plasma. The standard has been used in the field of blood and blood product safety, as well as in the clinical investigation of HBV infection, both for diagnosis and for monitoring HBV loads in response to antiviral therapy.

In the original collaborative study, three preparations were evaluated. Two of the materials were lyophilized preparations formulated by dilution of R1, the Eurohep reference material [2] in pooled human plasma. This plasma bulk containing HBV was stored at −70 °C until processing. A single bulk material was lyophilized on two separate occasions, 2 weeks apart, using the same processing parameters [1]. These lyophilized preparations, coded 97/746 and 97/750, were termed AA and BB, respectively, in the original collaborative study. A third preparation, termed CC, was a liquid/frozen HBV plasma sample. No significant difference in potency was observed between AA and BB, which had been prepared from the same bulk material, but had been lyophilized on separate occasions. The 1st IS for HBV DNA for NAT-based assays was assigned a potency of 10^6 international units per ml (10^6 IU/ml). In the 50th report of the WHO ECBS [3], it was noted that 97/750 would be reserved as a potential replacement standard in the future. As 97/750 had been fully characterized in the original collaborative study, the WHO ECBS proposed examination of real-time stability data of the 1st IS and the candidate replacement standard 97/750.

In the present collaborative study, the potency and stability of the candidate replacement standard 97/750 is compared to the 1st IS for HBV DNA. The approach for the re-evaluation of 97/750 was agreed on at the 16th meeting of the International Scientific Working Group on the Standardization of Genome Amplification Techniques (SoGAT) in May 2005 [4].

Materials and methods

The 1st IS for HBV DNA for NAT-based assays (97/746) and the proposed replacement (97/750) were lyophilized from the same bulk starting material derived from a high-titre HBV genotype A2 (HBV surface antigen subtype *adw2*) sample (Eurohep R1), diluted in human plasma [1]. This HBV strain has a sequence characteristic of those circulating in central Europe [2].

Collaborative study

Six laboratories participated in the collaborative study and each was requested to assay the 1st IS for HBV DNA (97/746) concurrently with the candidate replacement standard (97/750). The participating laboratories were from five different countries and represented quality control laboratories, a manufacturer of plasma derivatives and an academic institution (a national reference laboratory for hepatitis B and D). Participants were sent four vials of the 1st IS (97/746) and four vials of the candidate replacement standard (97/750), these were coded Samples 1 and 2. The normal temperature for the long-term storage of 97/746 and 97/750 is −20 °C and participants were requested to store the samples under these conditions until analysis. The aim of the study was to determine whether there was any evidence of loss of potency of the two lyophilized preparations during normal storage conditions, since the time they were freeze dried. Participants were requested to test the samples on four separate occasions. The lyophilized samples were reconstituted with 0·5 ml of nuclease-free deionized water and the contents gently agitated for 20 min before analysis. In the case of qualitative assays, participants were requested to perform serial dilutions of the samples in four independent assay runs. In the first qualitative assay run, 10-fold dilutions were performed to determine the end-point for the detection of HBV DNA. In each of the subsequent three assay runs, a minimum of two half-$\log_{10}$ dilutions either side of the predetermined end-point were tested, and results reported as positive or negative. In the case of quantitative assays for HBV DNA, participants were requested to report results in IU/ml and to test the samples without dilution, or prepare dilutions of the samples as necessary, depending on the linear range of assays used. In addition, one laboratory analysed the Eurohep R1 reference in parallel, following continuous storage at −80 °C.

Stability studies

For accelerated thermal degradation studies, vials of 97/746 and 97/750 were incubated at 4 °C and 20 °C, for between 51 months and 56 months. The degradation samples were extracted as previously described [5] and analysed in parallel with samples of the two preparations that had been stored at −20 °C, to provide a baseline for analysis. One set of assay runs was performed using the Artus HBV LC PCR Kit (Qiagen GmbH, Hilden, Germany) and used in accordance with the manufacturer's instructions. A second set of assay runs was performed using previously published primers and probe sequences [5] and amplification reactions were performed using the LightCycler FastStart DNA Master Hybprobe kit (Roche Applied Science, Mannheim, Germany). Standard curves were prepared using serial 10-fold dilutions of the 1st IS for HBV DNA (97/746). The stability studies were performed by two different operators at the National Institute for Biological Standards and Control (NIBSC), UK.