UK[30] and elsewhere[31], this model system may provide insight into this critical problem.

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# Cloned HBV DNA causes hepatitis in chimpanzees

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**Most of our knowledge of the structure and function of the hepatitis B virus (HBV) genome comes from the analysis of the viral DNA sequences cloned in bacteria[1–3]. Because the physical state of cloned HBV DNA differs from HBV DNA encapsidated in the virion—for example, it lacks the nick-gap structure[4], and a covalently linked protein[5]—the question arises as to whether it can initiate HBV replication *in vitro*[6] or *in vivo*. We describe here the development of typical acute viral hepatitis, and the detection of HBV-specific DNA sequences in the serum and liver, in a chimpanzee inoculated with cloned HBV DNA. This demonstrates that neither the virion proteins nor the nick-gap structure of the virion DNA are needed for the initiation of replication of HBV *in vivo*.**

**Table 1** Composition of the DNA mixture used for inoculation and transfection of chimpanzee

| Physical state | HBV DNA species | | |
|---|---|---|---|
| | HBV 14 | HBV 2 | HBV 6 |
| Tandem duplications | pSHH 14.3 (40 pmol) | pSHH 2.1 (8 pmol) | |
| Closed rings | pHBV 14.1 (8 pmol) | | pHBV 6 (6 pmol) |

Source, physical state and amount of HBV-containing plasmids in the DNA mixture used for inoculation of the chimpanzees. pHBV 14.1 contains a full length DNA fragment of HBV-14 DNA inserted into the *Pst* site of pBR322[10]. pSHH 14.3 contains a tandem duplication of the same DNA fragment in the *Pst*I site of the vector plasmid pSV010[11]. pSHH 2.1 contains HBV-2 DNA inserted as a tandem duplication into the *Eco*RI site of the vector plasmid pSV08[11]. pHBV6 contains the whole HBV-6 genome as an *Eco*RI fragment inserted into the *Eco*RI site of pBR325[12]. Closed HBV DNA circles were prepared by digestion of plasmids pHBV 14.1 and pHBV-6 with *Pst*I and *Eco*RI, respectively, and ligation with T4 ligase in conditions of preferential circularization of the HBV DNA fragments essentially as described[6]. The vector molecules were not removed from the DNA preparations as they do not replicate in animal cells, as holds true also for the pSV vectors that contain the origin of replication of SV40 but need large T antigen to replicate. The four DNAs were mixed in the ratio indicated, ethanol-precipitated and resuspended in 2 ml TNE (100 mM Tris *p*H 7.2, 155 mM NaCl, 0.1 mM EDTA). This DNA mixture was used for inoculation of the chimpanzee.

Three isolates of cloned HBV DNA derived from HBV of different serotypes were used in this study (Fig. 1), in an attempt to compensate by complementation or recombination for possible defects in the cloned DNA. The cloned HBV DNA was used in the form of closed circular and tandemly repeated molecules. A mixture of four of these DNA species (Table 1) was inoculated into a healthy chimpanzee intravenously, intramuscularly and directly into the liver. An autologous liver cell suspension transfected *in vitro* with the same DNA mixture was also inoculated into the liver. We adopted this approach of exposing the chimpanzee to a DNA mixture by different routes because no comparable experiments have previously been reported for HBV DNA, the chances for a successful outcome of such experiment seemed low, and also because of the limited number of available animals.

After inoculation, serum was regularly examined for liver function abnormalities, serological markers specific for HBV, and the presence of HBV DNA. Liver biopsies were examined histologically and for HBV DNA. Seven weeks after inoculation, the chimpanzee developed a typical, mild, self-limited, acute hepatitis. HBV surface antigen (HBsAg) subtype 'ay' (identified by subtype-specific antisera) appeared a week before an increase in the serum enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT). About 1–1$3\frac{1}{2}$ weeks later the first signs of the typical histology of a mild, acute hepatitis became recognizable in the liver biopsies (Table 2). On day 69, occasional portal mononuclear infiltration without obvious liver cell necrosis was seen. By day 81, a typical picture of acute viral hepatitis had developed. On day 101, resolving hepatitis was present. No further pathological changes were observed in a subsequent biopsy. HBV 'e' antigen (HBeAg) became detectable about 2 weeks after HBsAg, and both disappeared about 3 weeks later. The development of antibodies against HBsAg, HBeAg and HBV core antigen (HBcAg) and the kinetics of their appearance were normal. During the subsequent 9 months, there was no indication of the development of an HBV carrier state, or of chronic disease (serum bilirubin, serum enzymes and liver histology returned to and remained normal, and HBsAg and HBeAg did not persist), or of neoplastic liver disease (serum bilirubin, serum enzymes, $\alpha$-fetoprotein and carcinoembryonic antigen levels remained normal).

A similar pattern of disease was recorded in a repeat experiment with a second chimpanzee. In contrast, no signs of disease were observed in the year following the administration, by intravenous injection alone, of the same HBV mixture to another chimpanzee.

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To test whether the hepatitis was accompanied by replication of HBV DNA and the production of hepatitis B virus, DNA samples from blood and liver were analysed by the Southern technique[7] with a $^{32}$P-labelled, HBV-specific DNA probe. Sequences the size of viral DNA (3.2 kilobases (kb)) were present in the serum at the late, acute stage of the hepatitis (Fig. 2, lanes 1–3). Since the 3.2-kb band was much sharper after DNA repair synthesis the sequences were probably in the form of partially single-stranded virion DNA molecules. Cleavage with *Bgl*II yielded a major DNA species of 2.7 kb, as expected from HBV-14 or HBV-6 DNA circles but not from HBV2-DNA (see Fig. 1). Together with the result of the subtype determination (ay), this suggests that the major form of HBV DNA in the chimpanzee during hepatitis was HBV-14 DNA, the major species present in the mixture used for DNA transfer (Table 1).

The liver contained much more HBV DNA ($\sim 5 \times 10^8$ HBV DNA molecules per g of tissue) than serum ($\sim 10^6$ molecules ml$^{-1}$) and a more complex spectrum of HBV specific DNA molecules, which were predominantly non-integrated. They differed significantly from the DNA mixture used for transfection in the relative proportion of the different size molecules and in their restriction patterns (Fig. 2, lanes 7 to 12), indicating that a selective replication and possibly some rearrangement of the inoculated species of DNA had occurred. *Eco*RI and *Pst*I digestion both yielded a major 3.2-kb band (Fig. 2, lanes 9, 11), showing the presence of at least two of the inoculated DNA species (see Fig. 1). *Bgl*II produced a 2.3-kb DNA fragment, but not the 2.7-kb fragment produced from serum DNA (Fig. 2, lanes 3, 5), suggesting that HBV-2 was the predominant DNA species in the liver, but not the serum. Complex results were also obtained with other restriction endonucleases (data not shown), and we cannot draw any definite conclusion yet about the relationship between the several DNA species in the liver and the serum.

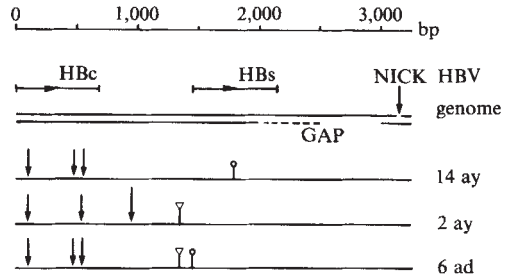


**Fig. 1** Features of the HBV genome and cloned HBV species used for transfection. The circular HBV genome has been opened at nucleotide 1 of the HBc gene. Positions and orientations of the genes for HBsAg and HBcAg are indicated on top using the numbering system of Pasek *et al.*[2]. For characterization of the HBV genomes used for transfection, three key enzyme cleavage maps are indicated (↓ *Bgl*II, ↑ *Pst*I, ⍑ *Eco*RI). The serotypes of the cloned HBV genomes as determined for the HBsAg produced in tissue culture cells, using SV40 expression vectors containing defined HBV DNA fragments, are also presented (H.W., unpublished). The three DNA species originate from independent clonings of HBV DNA. HBV-14 DNA was isolated from the HBV bank of Burrell *et al.*[17] by N. Gough. A restriction map of this clone has been described by N. Gough and K. Murray[21] and its complete HBV sequence has been determined[22]. HBV-2 DNA was originally isolated as a phage λ clone by Charnay *et al.*[18] and donated in plasmid pTKH10 by P. Tiollais. HBV-6 DNA originally cloned in pA01[19] was a gift of J. Summers.

That it was possible to induce the viral disease with all its usual clinical characteristics by inoculating cloned HBV DNA indicates that initiation of HBV replication requires—at least in these experimental conditions—neither the nick gap structure of the HBV genome as present in the virion (Dane particle) nor the virion proteins, including the viral DNA polymerase. That the hepatitis induced by cloned DNA followed a normal course is important because transfection of liver cells with DNA could have led to a different form of host cell–virus interaction. We are now trying to establish if the disease only develops after inoculation directly into the animal's liver, with or without

**Table 2** Course of hepatitis B after transfection with cloned HBV DNA

| | Laboratory determinations | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Days after infection | AST units | ALT units | HBsAg ng ml$^{-1}$ | Anti-HBs mIU | Anti-HBc titre | Anti-HBc IgM titre | HBeAg titre | Anti-HBe titre | Histology | DNA in: Serum | Liver |
| −52 | 23 | 36 | <0.1 | <1 | — | ND | — | — | ND | ND | ND |
| 0 | 21 | 33 | <0.1 | <1 | — | ND | — | — | ND | ND | ND |
| 11 | 18 | 41 | <0.1 | <1 | — | ND | — | — | ND | ND | ND |
| 18 | 15 | 15 | <0.1 | <1 | — | ND | — | — | ND | ND | ND |
| 25 | 15 | 55 | <0.1 | <1 | — | ND | — | — | ND | ND | ND |
| 32 | 18 | 23 | <0.1 | <1 | — | ND | — | — | ND | ND | ND |
| 39 | 20 | 15 | <0.1 | <1 | — | ND | — | — | ND | ND | ND |
| 46 | 21 | 25 | <0.6 | <1 | — | ND | — | — | ND | ND | ND |
| 53 | 60 | 80 | 5 | <1 | — | <100 | — | — | ND | ND | ND |
| 56 | 22 | 75 | 11 | <1 | — | <100 | — | — | − | ND | + |
| 62 | 14 | 30 | 182 | <1 | — | <100 | 43 | — | − | ND | + |
| 69 | ND | ND | 470 | <1 | 1 | <100 | 80 | — | (+) | − | + |
| 76 | 77 | 194 | <0.1 | 5 | 75 | 1,000 | — | + | + | + | + |
| 95 | 15 | 43 | <0.1 | 6 | 100 | 1,000 | — | + | ND | ND | ND |
| 101 | ND | ND | <0.1 | 7 | 70 | <100 | — | + | (+) | ND | ND |
| 109 | 18 | 23 | <0.1 | 11 | 45 | <100 | — | + | ND | ND | ND |
| 116 | 11 | 21 | <0.1 | 22 | 380 | <100 | — | + | ND | ND | ND |
| 179 | 15 | 33 | <0.1 | 141 | 400 | <100 | — | + | − | ND | ND |

Course of disease in a chimpanzee infected with cloned DNA. A colony-born, 3-yr old female was kept in specially designed isolation facilities. On day 0, 1 mg atropine and 160 mg ketamine hydrochloride were given, and anaesthesia was maintained with an oxygen–nitrous oxide–fluothane mixture. A surgical liver biopsy was taken. 500 μl each of the mixture of HBV DNAs described in Table 1 were inoculated intravenously and intramuscularly, and 900 μl directly into the liver. In addition, 900 μl of a liver cell suspension transfected previously *in vitro* with 100 μl of the same DNA mixture was inoculated into the liver. Transfection was carried out using DEAE dextran (molecular weight $2 \times 10^6$; Sigma) as a facilitator in a modification of the method described previously[13,14]. Liver biopsies (cylinders of liver tissue with a diameter of 1–2 mm, and a length of 9–15 mm) were taken from chimpanzees using Menghini needles, and transferred immediately into Williams medium (Flow Laboratories) containing 100 mM Tris-HCl *p*H 7.2 and 0.1 mM EDTA (WD-medium). The liver biopsies were washed three times with WD-medium and the cell suspension was prepared by mechanical disruption of the liver tissue. The liver cells were resuspended in 10 ml WD-medium, centrifuged for 5 min at 1,000 r.p.m. at room temperature, resuspended in 10 ml Williams medium (Flow Laboratories), recentrifuged for 5 min at 1,000 r.p.m. at room temperature and resuspended in 800 μl TNE buffer containing 100 μl 10×RPMI-1640 medium (Flow Laboratories). To this, 100 μl of the DNA mixture and 500 μg DEAE dextran dissolved in 100 μl TNE were added. The mixture was incubated for 1 h at 37 °C and reinjected into three different sites of the animal's liver under visual observation. All subsequent surgical or Menghini needle liver biopsies and bleedings were performed under general anaesthesia. AST, ALT, bilirubin and γ-glutamyl transpeptidase (γ-GT) were determined by standard techniques. Normal chimpanzee AST values: ≤40 units l$^{-1}$; normal chimpanzee ALT values: ≤40 units l$^{-1}$. Bilirubin and γ-GT levels did not exceed normal values (≤2 μM total bilirubin, and ≤35 units l$^{-1}$ γ-GT) throughout the study. HBsAg, anti-HBs, anti-HBc, anti-HBV IgM, HBeAg and anti-HBe were determined by commercially available (Abbott), or self-prepared[15,16] enzyme or radioimmunoassays. HBsAg was measured in a radioimmunoassay calibrated with a standard preparation. HBsAg subtypes were determined in the serum obtained 69 days after inoculation by blocking radioimmunoassays with monospecific 'ad' or 'ay' antibodies. Anti-HBs, anti-HBc and anti-HBc IgM were titrated as described previously[15]. HBeAg was titrated in a radioimmunoassay, and the reciprocal of the last serum dilution with a ratio of more than 2.1 of sample c.p.m.:negative control c.p.m. is given. Anti-HBe is indicated only as positive or negative. Serum (S) and liver (L) were tested for DNA; + or −, sample positive or negative for HBV DNA. Detailed results are given in the text and in Fig. 2 legend. ND, not determined.

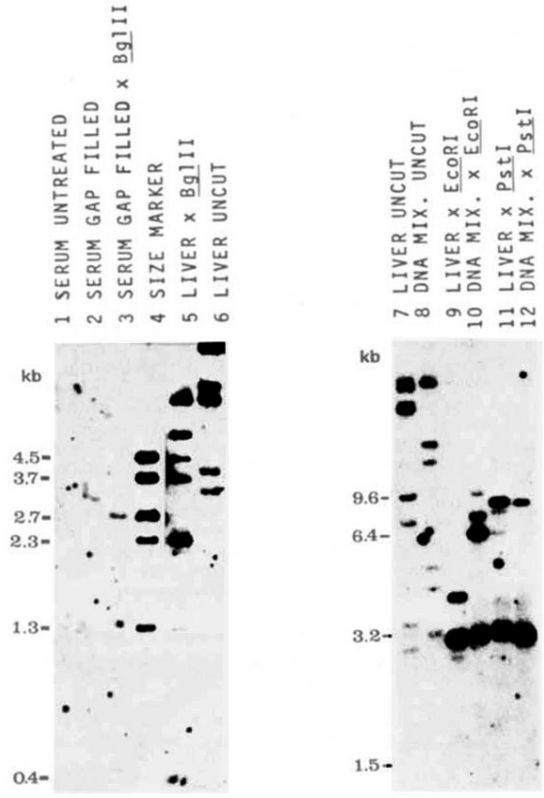


**Fig. 2** Southern blot analysis of HBV DNA present in chimpanzee serum and liver. Serum taken 76 days after inoculation was clarified by centrifugation for 20 min at 4 °C and 15,000 r.p.m. in a SW40 spinco rotor. The supernatant was recentrifuged for 3 h at 30,000 r.p.m. and the virus pellet sedimented through a 5–30% sucrose gradient at 35,000 r.p.m. for 12 h. DNA was isolated from the virus pellet using a proteinase K/SDS/phenol procedure[19,20], and part of the DNA was gap-filled with *Escherichia coli* DNA polymerase I. A piece of liver tissue from a surgical biopsy taken 69 days after transfection was minced, homogenized in TNE buffer and the DNA extracted as described above. For blot analysis samples corresponding to 1 ml serum or 20 μg liver DNA were loaded on two agarose gels before and after cleavage with restriction endonucleases. For the *Bgl*II analysis (lanes 3, 5) a *Bgl*II cleaved mixture of pSHH 14.3 and pSHH 2.1 was used as a reference in lane 4. The size marker for the second gel is not shown. Both gels were blotted onto nitrocellulose and hybridized with $^{32}$P-labelled HBV DNA isolated from pSHH 2.1 (specific activity $10^8$ c.p.m. per μg of DNA)[7]. Lanes 1–3 were exposed for 3 weeks, other lanes for shorter times.

transfection of liver cells *in vitro*, or whether it could be produced by intravenous inoculation. These experiments will provide more data about the possible risk of recombinant DNA molecules from animal viruses, and are expected to extend to a human pathogen those conclusions drawn previously from the polyoma/mouse model system[8,9].

The experimental system described here will also allow the identification *in vivo* of infectious HBV DNA molecules for development of cell or tissue culture systems for HBV replication. Meanwhile, this system will enable us to study *in vivo* the genetics of HBV using recombinant plasmids constructed *in vitro*, and to test different parts of the HBV DNA for their role in virus replication, their effect on host cells, and their contribution to the possible oncogenicity of the HBV genome.

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# Rearrangements between the immunoglobulin heavy chain gene $J_H$ and $C_\mu$ regions accompany normal B lymphocyte differentiation *in vitro*

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**Before B cells can synthesize their first immunoglobulin (Ig) molecules, membrane IgM, one of the many *V* regions, a *D* region, and a *J* region adjacent to the single $C_\mu$ region, must come together with the deletion of the intervening DNA[1–9]. Isotype switching during B-cell development is accompanied by reassociation of the fused *VDJ* complex with a $C_H$ region downstream from $C_\mu$. The exact points of reassociation are called switch sites, and have been located 5′ to each $C_H$ gene[10–12]. IgM-bearing B cells isolated from murine spleen showed no rearrangements in the area of switch sites between $J_H$ and $C_\mu$[7–9]. We now present results showing that the maturation of these cells to plasmablasts displaying isotype switching and immunoglobulin secretion in cultures containing bacterial lipopolysaccharide (LPS) is accompanied by DNA rearrangements between $J_H$ and $C_\mu$ sequences. As these plasmablasts arise in cultures of LPS stimulated cells that undergo few divisions, display IgM to IgG isotype switching, and evolve into secretory cells, it is suggested that these rearrangements may have a role in normal B-cell development.**

Rearrangements between $J_H$ and $C_\mu$ sequences were first detected among immunoglobulin-secreting hybridoma and plasmacytoma populations[5–7]. However, these rearrangements were largely dismissed due to the possibility that they represented tumour artefacts in these cell populations[9]. We therefore sought a system in which normal plasmablasts could be examined, and chose to analyse B lymphocytes after stimulation in culture with LPS[13]. An ethanol fixation technique allowed penetration of fluorochrome labelled anti-IgM into the cells without causing DNA damage or cell aggregation. Using this procedure, cells could be analysed and sorted according to their secretory IgM content with a fluorescence activated cell sorter (FACS). Figure 1*a* shows a size distribution of cells stimulated *in vitro* for 4 days with LPS, and stained for cytoplasmic IgM. Figure 1*b* shows the fluorescence distribution of the sized population indicated by arrows in Fig. 1*a*. The plot indicates that 20% of the cells are negative, while 80% compose a distinct fluorescent population showing staining with the anti-IgM reagent.

The negative population was not apparent on analysis of similar cultures treated with anti-Thy 1.2 plus complement to remove T cells and then recultured overnight to remove adherent and dead cells before fixation and staining (Fig. 1*c*). This finding suggests that most LPS-stimulated B cells develop at

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