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# Experimental transfection of *Macaca sylvanus* with cloned human hepatitis B virus

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**Due to the absence of easily accessible animal models for the study of hepatitis B virus (HBV), the possibility of using *Macaca sylvanus*, a monkey originating from Morocco, North Africa, was investigated. Three monkeys were intrahepatically inoculated with a replication-competent head-to-tail HBV DNA plasmid dimer construct. The HBV surface antigen and HBV DNA were detected prior to alanine aminotransferase elevation in the serum of two of three HBV-inoculated monkeys at day 2 post-transfection and persisted for several weeks. This indicates that transfected animals developed markers of HBV infection. In addition, electron microscopy of the serum 3 weeks post-transfection showed the presence of virus particles whose shape and size were similar to complete 42 nm HBV Dane particles. Histological examination of liver tissues also revealed pathological changes not observed in uninfected controls, which strongly suggested acute hepatitis. HBV DNA was also detected by PCR in these monkey livers. Taken together, these results indicate that HBV can successfully replicate in this model and that *M. sylvanus* could be a potentially useful new primate model for the study of HBV replication.**

In spite of the availability of an effective vaccine, infection by hepatitis B virus (HBV) remains a worldwide public health problem, with 400 million chronic HBV carriers. Every year, nearly 1 million individuals succumb to HBV-associated liver disease, especially cirrhosis and hepatocellular carcinoma (Wright *et al.*, 1993). The treatment of chronic HBV infection is still unsatisfactory. Therefore, the development of better animal models to test new therapeutic approaches is highly desirable. Nowadays, several nonprimate animal models, which are naturally infected by HBV-related hepadnaviruses, are available for *in vivo* screening of putative antiviral compounds. Pekin ducks infected by duck HBV (Lambert *et al.*, 1991; Cova *et al.*, 1993; Le Guerhier *et al.*, 2000) and American woodchuck infected by woodchuck HBV (Hantz *et al.*, 1984) are the two models currently used to test the effectiveness of new therapeutic approaches, including screening of new antiviral molecules (Zoulim & Trepo, 1999) or evaluation of DNA-based vaccines (Davis *et al.*, 1996; Donnelly *et al.*, 1997; Rollier *et al.*, 1999). The development of a new experimental model that is closer to humans and susceptible to HBV infection is of particular importance, since it will represent an essential tool for drug and HBV variant testing. Although chimpanzees can be readily infected by HBV and develop acute hepatitis (Will *et al.*, 1982), this species is endangered and expensive and, thus, is inappropriate for research programs that require a large number of animals. Attempts of experimental *in vivo* HBV infections of tupaias (Walter *et al.*, 1996; Yan *et al.*, 1996) and baboons (Kedda *et al.*, 2000) were also performed, although transient infection of these animals did result in rapid seroconversion.

Several seroepidemiological studies in monkeys indicate that many old world species, such as macaques, chimpanzees, African green monkeys and baboons, are simian T-cell leukaemia virus type 1 carriers (Ibrahim *et al.*, 1995; Ishikawa *et al.*, 1987; Mahieux *et al.*, 1998; Meertens *et al.*, 2001). These studies suggest that lymphotropic and also hepatotropic viruses may be transmitted between different African monkey species but also from monkeys to humans.

In the present study, we have evaluated the potential use of *Macaca sylvanus* as a new experimental and alternative primate model for HBV infection. These monkeys were captured in the wild (middle Atlas mountains) and were quarantined and

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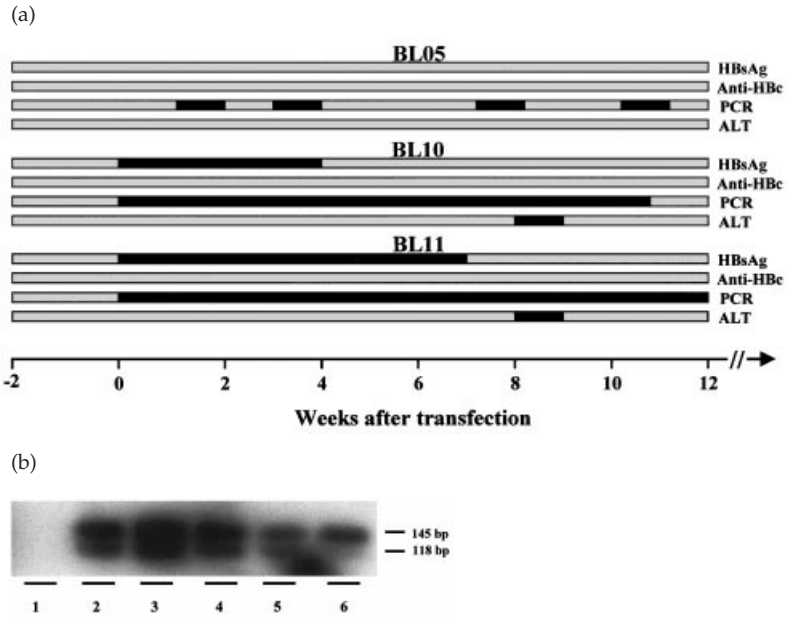


Fig. 1. (a) Schematic representation of the follow-up of markers of HBV infection in animals BL05, BL10 and BL11. HBsAg detection was performed with the bioMérieux VIDAS HBsAg Ultradetection kit and the ORTHO antibody to HBsAg ELISA, system 3. The limit of detection of such tests is about 0·2–0·3 ng/ml. Total anti-HBc antibody detection was performed with the Vidas Anti-HBc Total II kit (bioMérieux). ALT levels were determined using a Welltech kit. HBV DNA was detected by PCR in the S and C genes, as described in the text. Shading in the horizontal bars represents a positive response for the corresponding parameter. (b) Southern blot analysis of PCR products (145 and 118 bp) located in the S and C genes. Lanes 2–6 represent PCR detection of HBV DNA in animal BL11 at weeks 1, 2, 3, 4 and 7, respectively. Lane 1 corresponds to a serum sample from animal BL11 prior to inoculation (used as a control).

maintained at the Pasteur Institute of Casablanca (Morocco) under conditions that met or exceeded all of the requirements needed for the physical and psychological well being of such animals. These animals had not been exposed to any hepatotropic viruses prior to the *in vivo* inoculation of HBV DNA and all animals were negative for serological markers of infection with hepatitis A, B and C viruses as well as human T-lymphotropic viruses types I and II. We established the baseline of alanine aminotransferase (ALT) at 61 U/L by calculating the mean of all of the sera sampled from all of the animals over a period of 3 months before inoculation of HBV DNA. This value of 61 U/L is in accordance with ALT levels in humans (56 U/L) and chimpanzees (55 U/L) (Bassett *et al.*, 1999). All molecular analyses were performed at INSERM U271 (Lyon, France). Three *M. sylvanus* monkeys (BL05, BL10 and BL11) were intrahepatically inoculated with a total of 450 μg of a replication-competent head-to-tail HBV DNA (ayw, genotype D) plasmid dimer (pBR322) construct (Burrell *et al.*, 1979; Charnay *et al.*, 1979; Galibert *et al.*, 1979) dissolved in water and injected into three sites of the liver using paediatric Menghini needles, as described for chimpanzees (Will *et al.*, 1982), tupaias (Walter *et al.*, 1996) and ducks (Sprengel *et al.*, 1984). After inoculation, animals were bled weekly to test for HBV surface antigens (HBsAg), anti-HBc antibodies and ALT and aminotransferase (AST) activities. Out of the three control *M. sylvanus* monkeys (BL15, BL16 and BL17), one died accidentally prior to the beginning of the experiment. After 36 weeks of follow-up, the animals were sacrificed. All experiments were carried out under general anaesthesia using ketamine (1 mg/kg body weight) (IMALGEN).

Interestingly, two of three *M. sylvanus* monkeys transfected with cloned HBV DNA developed markers of virus infection, while the two control animals followed in parallel didn't develop such markers. For one animal (BL05) from the HBV DNA-transfected group, no serum HBsAg was detected. In contrast, starting 2 days post-transfection, HBsAg expression was detected in the serum of monkeys BL10 and BL11 for a period of 4 and 7 weeks, respectively (Fig. 1a). Similar to acute self-limited HBV in humans, HBsAg was eliminated from the serum of these monkeys. Therefore, in *M. sylvanus*, HBsAg positivity persisted for a longer time (4–7 weeks) than for experimental HBV infection in tupaias or some chimpanzees (Will *et al.*, 1982; Walter *et al.*, 1996), which revealed only transient HBsAg expression in serum that was limited to a few days and up to a maximum of 4 weeks.

After transfection, no anti-HBc antibodies were detected in the serum of *M. sylvanus* monkeys. This may occur due to either the low level of anti-HBc antibodies in the serum of the animals or the lack of reactivity of anti-HBc antibodies elicited in *M. sylvanus* monkeys. It is of note that the test used (bioMérieux Vidas Anti-HBc Detection kit) is designed for detection in human sera. Also, anti-HBsAg antibodies were not detected, probably for the same reason. We have not looked

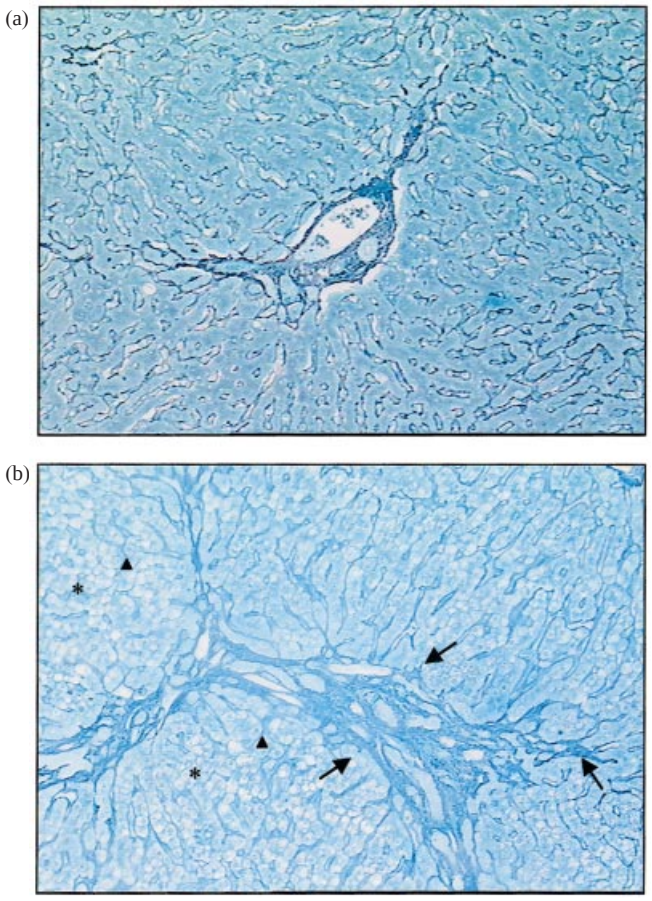


Fig. 2. Histological analysis of liver biopsies after reticulin staining. (a) Normal portal tract and regular reticulin patterns in the lobule of an uninfected animal. (b) Increase in the size of the portal tract, early periportal fibrosis (arrows) and disorganized cell plate patterns (asterisk) in the lobule of an HBV DNA-transfected animal, BL11. Note the hepatocyte swelling (arrowheads). Original magnification, 10×.

for the HVB e-antigen because of the limited amount of serum available.

We also tested for the presence of HBV DNA in the sera of transfected *M. sylvanus* monkeys using PCR and Southern blot analysis (Fig. 1a). Primers for PCR amplification were selected from a domain overlapping the core and the surface gene, which is highly conserved among all human HBV genotypes. Both a 118 bp region in the surface gene (sense, 5′ GGAGTG-GGCCTCAGCCCGTTTCTC 3′; reverse, 5′ GCCCCCAAT-ACCACATCATCCATA 3′) and a 145 bp region in the core gene (sense, 5′ TCGGAGTGTGGATTCGCACTCCTC 3′; reverse, 5′ GATTGAGACCTTCCTCCTCTGCGAGGA 3′) were amplified. The specificity of the amplified bands was confirmed by Southern blot hybridization using a $^{32}$P-labelled random-primed HBV DNA probe. High stringency hybridization conditions using buffers containing 50% formamide, 7% SDS, 0·25 M sodium phosphate, pH 7·2, 0·25 M NaCl and 1 mM EDTA were used at 42 °C with washes in 2–0·5 × SSC containing 0·1% SDS at 65 °C. The results showed significant and persistent expression of viral DNA during follow-up for both *M. sylvanus* monkeys BL10 and BL11, which was particularly intense for BL11, as illustrated in Fig. 1(b). In order to quantify HBV DNA in the serum of *M. sylvanus* BL11, quantitative PCR was performed using the Amplicor HBV Monitor test (Roche). Primers used in this test are located within the HBV preC/C region and the sensitivity of the test has been estimated to range between $10^3$ and $10^{5·5}$ genome copies/ml. A progressive increase in serum HBV genome copy numbers was observed during the first 3 weeks post HBV DNA inoculation, which peaked at $2·10^5$ genome copies/ml at week 3 (data not shown).

In addition, a transient and significant peak of transaminase activity was observed for animals BL10 (307 U/L) and BL11 (286 U/L) at week 8 but no increase was observed for BL05 (Fig. 1a). Moreover, after HBV DNA inoculation, biochemical evidence of hepatitis was correlated with HBV DNA detection by PCR and abnormal histological findings. As illustrated in Fig. 2(b), histological analysis of liver sections from these animals revealed definite modifications of infected hepatocytes, including swelling of the cells and disarray of cell plates but without cell necrosis. Such changes were conspicuous in both the lobules and portal tract. Early portal fibrosis could be demonstrated by the reticulin stain, which revealed slightly enlarged portal tracts extending in thin periportal septa. As illustrated in the Fig. 2(a), liver analysis from control animals did not show such abnormalities and no rise in serum transaminase levels was seen during follow-up.

During the course of the experiment, we were not able to perform biopsies of the animals enrolled in the study. Nevertheless, to search for intrahepatic HBV replication, Southern blot analysis of total intrahepatic DNA from animals BL10 and BL11 was performed using autopsy liver samples obtained at the end of the experiment, i.e. 9 months after the initial HBV DNA inoculation. Despite intrahepatic HBV DNA detection by PCR, Southern blot analysis of liver samples from these animals did not reveal the presence of HBV DNA replicative forms. The low level of HBV DNA replication in the liver might explain this. Similarly, the replicative forms of HBV DNA were not detected in the livers of HBV DNA-transfected chimpanzee (Will *et al.*, 1983).

We have also looked for the presence of virus particles in the serum of monkey BL11 serum using sucrose gradient sedimentation followed by electron microscopy examination. To this end, serum samples (1·5 ml) from this animal, which were HBsAg- and HBV DNA-positive ($2·10^5$ genome copies/ml of serum) at week 3 post-transfection, had first been concentrated by centrifuging at 210000 ***g*** for 2 h in a Beckman L8-70M ultracentrifuge. After serum ultracentrifugation, the pellet was resuspended in 150 µl PBS and subjected to equilibrium centrifugation on a 15–45% (w/w) linear sucrose gradient in PBS buffer for 17 h at 210000 ***g*** at 4 °C. HBV DNA was analysed by PCR and Southern blotting following nucleic acid extraction in all collected fractions. HBV DNA was

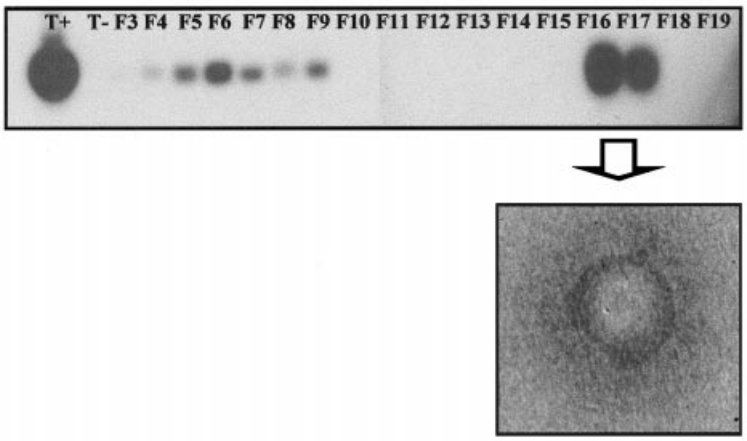


Fig. 3. Isopycnic sucrose gradient ultracentrifugation was performed on 1·5 ml of serum from a transfected *M. sylvanus* monkey, BL11, positive for HBsAg and HBV DNA. A total of 20 fractions was collected and tested for HBV DNA by PCR specific for the S gene. A Southern blot of fractions 3–19 (F3–F19) is shown. Fractions were absorbed onto carbon-coated grids for 10 min, rinsed in water for 2 min and stained with 4% APT, pH 7·2, and dried on absorbent paper prior to examined by a JEOL 100 CX electron microscope. The presence of a 42 nm diameter virus particle in the pooled fractions 16 and 17 is shown below.

detected between 22 and 27·5 % sucrose (fractions 3–9). It was generally admitted that such virus particles did not contain HBV DNA when HBV DNA was detected by the classical hybridization technique. However, when we used highly sensitive PCR techniques, especially nested PCR or PCR followed by Southern blotting, HBV DNA could be detected in fractions at 20–25 % sucrose, as described by Petit *et al.* (2001).

A more intense signal was found between 40 and 41·5 % sucrose (fractions 16 and 17) (Fig. 3). Electron microscopy examination of these fractions, 16 and 17, further purified by dialysis and ultracentrifugation, revealed the presence of incomplete 22 nm particles in fraction 6 (25 % sucrose) (data not shown) as well as 42 nm virions in fraction 17 (41·5 % sucrose) (Fig. 3). The size and shape of virions in fraction 17 was similar to complete HBV Dane particles, which have been previously identified at a similar density (42 % sucrose) following equilibrium centrifugation of HBV DNA-positive human serum under the same centrifugation conditions (Petit *et al.*, 1990). Sucrose gradient fractions remaining negative for HBV DNA by PCR did not exhibit the presence of any virus-like particles. Therefore, our results strongly suggest that complete HBV particles can be produced in the serum of HBV-transfected *M. sylvanus* monkeys.

Importantly, the histological examination of liver tissues from *M. sylvanus* animals transfected by HBV DNA demonstrated pathological changes, which strongly suggested that acute infection did occur in the *M. sylvanus* animal model. Indeed, the pattern of liver injury at necropsy was suggestive of regeneration associated with the resolutive phase of acute hepatitis. The minimal fibrosis observed was integrated into this interpretation. In contrast, no evidence of hepatic injury was shown by histological examination of the liver tissue from HBV-infected tupaias (Walter *et al.*, 1996) or baboons (Kedda *et al.*, 2000) and no elevated serum AST levels were observed in these animals.

As described for tupaias (Yan *et al.*, 1996), serial passages of released HBV particles will be attempted in *M. sylvanus* monkeys to test their infectivity and to favour virus infection in this model. This may improve the efficiency of infection, allow higher levels of viral gene expression and may lead to the establishment of a chronic infection.

Taken together, our results show that, following direct HBV DNA transfection of *M. sylvanus* liver, we have been able to detect in the serum of two of three transfected animals, increasing amounts of circulating HBV DNA together with the presence of HBsAg. In addition, serum ultracentrifugation experiments did confirm the presence of virus particles, with a size and density characteristic of HBV Dane particles observed only in the HBV DNA-positive fractions. Moreover, the presence of virus markers in the sera of transfected monkeys was followed by increased transaminase levels and histological patterns, suggestive of resolving acute liver injury. Therefore, our study demonstrates for the first time the occurrence of HBV replication associated with acute hepatitis after intra-hepatic transfection of *M. sylvanus* monkeys with HBV DNA.

In conclusion, our study demonstrates that *M. sylvanus*, a small monkey abundant in Morocco, could be a potentially useful new primate model for the study of HBV replication. If we succeed through serial passage of HBV in *M. sylvanus* and, possibly, in developing an immunosuppressive regimen to establish a chronic HBV infection, such a model will be suitable for the study of the safety, immunogenicity and efficacy of novel HBV therapeutic vaccines as well as antiviral strategies but also for the testing of replication capacity of HBV mutants that emerge in chronically infected patients.

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## References

**Bassett, S. E., Di Bisceglie, A. M., Bacon, B. R., Sharp, R. M., Govindarajan, S., Hubbard, G. B., Brasky, K. M. & Lanford, R. E. (1999).** Effects of iron loading on pathogenicity in hepatitis C virus-infected chimpanzees. *Hepatology* **29**, 1884–1892.

**Burrell, C. J., Mackay, P., Greenaway, P. J., Hofschneider, P. H. & Murray, K. (1979).** Expression in *Escherichia coli* of hepatitis B virus DNA sequences cloned in plasmid pBR322. *Nature* **279**, 43–47.

**Charnay, P., Pourcel, C., Louise, A., Fritsch, A. & Tiollais, P. (1979).** Cloning in *Escherichia coli* and physical structure of hepatitis B virion DNA. *Proceedings of the National Academy of Sciences, USA* **76**, 2222–2226.

**Cova, L., Fourel, I., Vitvitski, L., Lambert, V., Chassot, S., Hantz, O. & Trepo, C. (1993).** Animal models for the understanding and control of HBV and HDV infections. *Journal of Hepatology* **17** (Suppl. 3), S143–S148.

**Davis, H. L., McCluskie, M. J., Gerin, J. L. & Purcell, R. H. (1996).** DNA vaccine for hepatitis B: evidence for immunogenicity in chimpanzees and comparison with other vaccines. *Proceedings of the National Academy of Sciences, USA* **93**, 7213–7218.

**Donnelly, J. J., Ulmer, J. B. & Liu, M. A. (1997).** DNA vaccines. *Life Sciences* **60**, 163–172.

**Galibert, F., Mandart, E., Fitoussi, F., Tiollais, P. & Charnay, P. (1979).** Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. coli*. *Nature* **281**, 646–650.

**Hantz, O., Ooka, T., Vitvitski, L., Pichoud, C. & Trepo, C. (1984).** Comparison of properties of woodchuck hepatitis virus and human hepatitis B virus endogenous DNA polymerases. *Antimicrobial Agents and Chemotherapy* **25**, 242–246.

**Ibrahim, F., de The, G. & Gessain, A. (1995).** Isolation and characterization of a new simian T-cell leukemia virus type 1 from naturally infected celebes macaques (*Macaca tonkeana*): complete nucleotide sequence and phylogenetic relationship with the Australo-Melanesian human T-cell leukemia virus type 1. *Journal of Virology* **69**, 6980–6993.

**Ishikawa, K., Fukasawa, M., Tsujimoto, H., Else, J. G., Isahakia, M., Ubhi, N. K., Ishida, T., Takenaka, O., Kawamoto, Y., Shotake, T. and others (1987).** Serological survey and virus isolation of simian T-cell leukemia/T-lymphotropic virus type I (STLV-I) in non-human primates in their native countries. *International Journal of Cancer* **40**, 233–239.

**Kedda, M. A., Kramvis, A., Kew, M. C., Lecatsas, G., Paterson, A. C., Aspinall, S., Stark, J. H., De Klerk, W. A. & Gridelli, B. (2000).** Susceptibility of chacma baboons (*Papio ursinus orientalis*) to infection by hepatitis B virus. *Transplantation* **69**, 1429–1434.

**Lambert, V., Chassot, S., Kay, A., Trepo, C. & Cova, L. (1991).** *In vivo* neutralization of duck hepatitis B virus by antibodies specific to the N-terminal portion of pre-S protein. *Virology* **185**, 446–450.

**Le Guerhier, F., Pichoud, C., Guerret, S., Chevallier, M., Jamard, C., Hantz, O., Li, X. Y., Chen, S. H., King, I., Trepo, C., Cheng, Y. C. & Zoulim, F. (2000).** Characterization of the antiviral effect of 2′,3′-dideoxy-2′,3′-didehydro-β-L-5-fluorocytidine in the duck hepatitis B virus infection model. *Antimicrobial Agents and Chemotherapy* **44**, 111–122.

**Mahieux, R., Pecon-Slattery, J., Chen, G. M. & Gessain, A. (1998).** Evolutionary inferences of novel simian T lymphotropic virus type 1 from wild-caught chacma (*Papio ursinus*) and olive baboons (*Papio anubis*). *Virology* **251**, 71–84.

**Meertens, L., Rigoulet, J., Mauclere, P., Van Beveren, M., Chen, G. M., Diop, O., Dubreuil, G., Georges-Goubot, M. C., Berthier, J. L., Lewis, J. & Gessain, A. (2001).** Molecular and phylogenetic analyses of 16 novel simian T cell leukemia virus type 1 from Africa: close relationship of STLV-1 from *Allenopithecus nigroviridis* to HTLV-1 subtype B strains. *Virology* **287**, 275–285.

**Petit, M. A., Zoulim, F., Capel, F., Dubanchet, S., Dauguet, C. & Trepo, C. (1990).** Variable expression of preS1 antigen in serum during chronic hepatitis B virus infection: an accurate marker for the level of hepatitis B virus replication. *Hepatology* **11**, 809–814.

**Petit, M. A., Buffello-Le Guillou, D., Roche, B., Dussaix, E., Duclos-Vallee, J. C., Feray, C. & Samuel, D. (2001).** Residual hepatitis B virus particles in liver transplant recipients receiving lamivudine: PCR quantitation of HBV DNA and ELISA of preS1 antigen. *Journal of Medical Virology* **65**, 493–504.

**Rollier, C., Sunyach, C., Barraud, L., Madani, N., Jamard, C., Trepo, C. & Cova, L. (1999).** Protective and therapeutic effect of DNA-based immunization against hepadnavirus large envelope protein. *Gastroenterology* **116**, 658–665.

**Sprengel, R., Kuhn, C., Manso, C. & Will, H. (1984).** Cloned duck hepatitis B virus DNA is infectious in Pekin ducks. *Journal of Virology* **52**, 932–937.

**Walter, E., Keist, R., Niederost, B., Pult, I. & Blum, H. E. (1996).** Hepatitis B virus infection of tupaia hepatocytes *in vitro* and *in vivo*. *Hepatology* **24**, 1–5.

**Will, H., Cattaneo, R., Koch, H. G., Darai, G., Schaller, H., Schellekens, H., van Eerd, P. M. & Deinhardt, F. (1982).** Cloned HBV DNA causes hepatitis in chimpanzees. *Nature* **299**, 740–742.

**Will, H., Darai, G., Deinhardt, F., Schellekens, H. & Schaller, H. (1983).** Hepatitis B after infection of a chimpanzee with cloned HBV DNA. *Developments in Biological Standardization* **54**, 131–133.

**Wright, T. L. & Lau, J. Y. (1993).** Clinical aspects of hepatitis B virus infection. *Lancet* **342**, 1340–1344.

**Yan, R. Q., Su, J. J., Huang, D. R., Gan, Y. C., Yang, C. & Huang, G. H. (1996).** Human hepatitis B virus and hepatocellular carcinoma. I. Experimental infection of tree shrews with hepatitis B virus. *Journal of Cancer Research and Clinical Oncology* **122**, 283–288.

**Zoulim, F. & Trepo, C. (1999).** New antiviral agents for the therapy of chronic hepatitis B virus infection. *Intervirology* **42**, 125–144.

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