Requirement of Activation for Hepatitis B Virus Infection

by

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24 **Abstract**

25 Although *in vitro* models of human hepatitis B virus replication are established, so far
26 none could approximate infection efficiency as expected from *in vivo* observations.
27 Susceptibility for HBV infections has only been reported for primary hepatocytes of
28 human, chimpanzee or *Tupaia belangeri* and the cell line HepaRG. Here we show that the
29 insusceptible human hepatoma cell line HepG2 can be infected, when the virus was
30 beforehand activated by passage over whole duck liver cell cultures. That suggests an
31 activation step to be performed by specialized liver cells.

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47 The hepatitis B virus (HBV) is a small single stranded DNA-containing enveloped virus.
 48 Depending on its relatively strict organotropicity HBV infects mainly hepatocytes and
 49 causes severe liver diseases in humans ¹. For this reason it was so far only possible to
 50 infect *in vitro* cultures of primary human or Tupaia belangeri hepatocytes ^{2,3}, whereas
 51 human hepatoma cell lines are usually refractory to HBV infection. We succeeded in
 52 infecting the hepatoma cell line HepG2 with HBV by ‘activating’ the virus in a culture of
 53 primary duck liver cells (PDLcs).

54 Using duck HBV (DHBV)-infected PDLcs as a model data were published indicating
 55 that eventually scavenging liver sinusoidal endothelial cells (LSECs) play the key role in
 56 the initial uptake and targeting of hepadnaviruses in the liver ⁴. These data implicated that
 57 in the process of transcytosis through LSECs the viral surface of HBV may be altered as
 58 a prerequisite for the infection procedure, a step that may be limiting for the infection of
 59 hepatoma cell lines like HepG2 (Fig. 1b). Further *in vitro* experiments performed in our
 60 laboratory using preparations of embryonal PDLcs ^{5,6} infected with purified DHBV
 61 virions ⁷ revealed that at least co-staining of single LSECs could be measured using
 62 fluorescence-labeled acetylated low density lipoprotein for the identification of LSECs
 63 and anti-DHBc antibodies for the localization of the virions (not shown). Taking into
 64 account a possibly high conservation of LSEC function we predicted that the human
 65 HBV could also be found in association with LSECs in a duck liver cell culture. Indeed,
 66 immunofluorescence studies performed with rabbit antisera against HBc or the human
 67 nuclear factor of activated T cells (NFATc1; Santa Cruz) as a marker of LSECs revealed
 68 24 h after the addition of HBV, similarly as described for DHBV-infected PDLcs ⁸, co-
 69 localization of HBV and NFATc1 in a PDLc culture (Fig. 1a). Cells, negative for

70 NFATc1 but strongly reacting with anti-HBc antiserum, are possibly Kupffer cells with
 71 their typical phagocytic activity. As a source of HBV virions, HepG2.2.15 cells, HepG2
 72 cells stably transfected with cloned HBV-DNA, were utilized for the production of
 73 infectious HBV ⁹, which was concentrated 50-fold from the collected supernatants ¹⁰.
 74 The calculation of virions was performed by DNA dot blot, using a gel-purified full-
 75 length *EcoRI* fragment of cloned HBV DNA labeled with [α -³²P] dCTP as a probe ⁶.
 76 When we measured the amount of virions after the passage through a PDLC culture,
 77 designated as HBV*, against the initial amount of HBV, we found a reduction of about
 78 60 %, supposedly lost by unspecific adsorption and phagocytosis (Fig. 1c). In order to
 79 test the infectivity of HBV* for HepG2 cells after passage through primary duck LSECs,
 80 a time course kinetic for the release of virions after infection was performed (Fig. 2a). It
 81 revealed a steady increase of the amount of viral DNA (again measured by dot blot) in
 82 the supernatants of HepG2 cells, comparable to the cell growth-dependent increase of
 83 viral output in the stably transfected hepatoma cell line HepG2.2.15. Otherwise, no viral
 84 DNA could be detected in the supernatants when untreated HBV was used directly for
 85 infection. To study viral DNA replication in infected HepG2 cells in more details we
 86 performed a Southern blot using the same radioactively labeled probe as described above.
 87 Besides the full-length relaxed circular DNA single-stranded replicative intermediates of
 88 HBV DNA were also detected (Fig. 2b). In this study we could find out three further
 89 details: first, an incubation time of HBV with LSECs of 24 h instead of 4 h did not lead to
 90 an increase of infectivity; second, transfer of HBV-incubated activator cells instead of
 91 activated virus was also able to initiate HBV-infection; third, to employ another cell type,
 92 primary duck kidney cells ⁸, as activator cells would not support subsequent infection of

93 hepatoma cell lines. In a next experiment we replaced every 24 h the culture medium of
94 HBV infected HepG2 cells for the calculation of the amount of the viral surface proteins
95 with a commercially available anti-HBs-coated micro particle enzyme immunoassay
96 (Abbott AXSYM system, Germany) as a measure of virus production per day (Fig. 2c).
97 Seven days after infection the cells were disrupted to reveal HBV proteins after
98 electrophoretical separation over a 5 to 20 % polyacrylamide gradient by Western blots
99 with rabbit antisera raised against the surface proteins (HBs) or the core protein (HBc) of
100 HBV as described ⁶. Only in HBV*-infected cultures the synthesis of HBV core and
101 surface proteins could be demonstrated by immunoblotting (Fig. 2d). The alteration HBV
102 virions acquire during passage through PDLC cultures would supposedly concern
103 envelope protein integrity or reversible modification. We therefore compared their
104 appearance in immunoblotting. The general glycosylation pattern of the viral envelope
105 proteins of HBV and HBV* was unchanged. Yet, we found the amount of the
106 unglycosylated small surface protein of HBV to be significantly reduced; whereas the
107 glycosylated as well as the unglycosylated middle and large surface proteins did not show
108 particular alterations (Fig. 1d).

109 We conclude that hepatoma cell lines like HepG2 are susceptible to infection with HBV
110 after a yet to define activation step for HBV virions that takes place in whole liver cell
111 cultures and may even play a significant role in physiological HBV infections. The main
112 suspect for HBV activation would be LSECs as they constitute a permeable barrier
113 between the blood stream and the liver parenchyma and thus necessarily come into close
114 contact with trespassing particles ¹¹. The absence of morphological features comparable
115 to the liver architecture in cell cultures or defined conditions for the maintenance of

specialized cell types may be the major player that hampers *in vitro* hepadnaviral infections. Another possibility to influence infectivity of HBV virions would only be secretion of relevant molecules by a cell type in the culture. The establishment of pure LSEC cultures or LSEC cell lines and close pursuance of viral particles within the cells will permit to throw light on their participation in the proposed viral activation.

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References

1. Ganem, D. in *Fields Virology* (eds. Fields, B. N., Knipe, D. M. & Howley, P. M.) 2703-2737 (Lippincott-Raven, Philadelphia, 1996).
2. Gripon, P. et al. Hepatitis B virus infection of adult human hepatocytes cultured in the presence of dimethyl sulfoxide. *J Virol* **62**, 4136-43 (1988).
3. Walter, E., Keist, R., Niederost, B., Pult, I. & Blum, H. E. Hepatitis B virus infection of tupaia hepatocytes in vitro and in vivo. *Hepatology* **24**, 1-5 (1996).
4. Breiner, K. M., Schaller, H. & Knolle, P. A. Endothelial cell-mediated uptake of a hepatitis B virus: a new concept of liver targeting of hepatotropic microorganisms. *Hepatology* **34**, 803-8 (2001).
5. Kock, J. & Schlicht, H. J. Analysis of the earliest steps of hepadnavirus replication: genome repair after infectious entry into hepatocytes does not depend on viral polymerase activity. *J Virol* **67**, 4867-74 (1993).
6. Bruns, M., Miska, S., Chassot, S. & Will, H. Enhancement of hepatitis B virus infection by noninfectious subviral particles. *J Virol* **72**, 1462-8 (1998).
7. Franke, C., Matschl, U. & Bruns, M. Enzymatic treatment of duck hepatitis B virus: Topology of the surface proteins for virions and noninfectious subviral particles. *Virology* **359**, 126-137 (2007).
8. Maenz, C., Chang, S.-F., Iwanski, A. & Bruns, M. Entry of duck hepatitis B virus into primary duck liver and kidney cells after discovery of a fusogenic region within the large surface protein. *J Virol* **81**, 5014-5023 (2007).
9. Sells, M. A., Chen, M. L. & Acs, G. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proc Natl Acad Sci U S A* **84**, 1005-9 (1987).
10. Gripon, P. et al. Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci U S A* **99**, 15655-60 (2002).
11. Wisse, E., De Zanger, R. B., Charels, K., Van Der Smissen, P. & McCuskey, R. S. The liver sieve: considerations concerning the structure and function of endothelial fenestrae, the sinusoidal wall and the space of Disse. *Hepatology* **5**, 683-92 (1985).

Figure Legends

Fig. 1. Activation of HBV by PDLCs for infection of human hepatoma cells. (a) Co-immunofluorescence staining of PDLCs after incubation with HBV using anti-HBc and anti-NFATc1 antisera. (b) Model to demonstrate the requirement for activation of HBV by PDLCs before the infection of target cells like HepG2. (c) Analysis of HBV DNA before and after the addition to PDLCs. HBV was added to PDLCs and removed again after an incubation time of 4 h. Calculation of the viral DNAs before and after incubation with PDLCs revealed a loss of HBV virions of about 60 %; the presented dot blot data shows the mean of 3 measurements. (d) The same amount of virus particles was used to investigate changes of the viral envelope proteins before and after incubation with PDLCs; GP, glycoprotein, and P, protein, indicate the molecular weight in kD of the HBV large (L), middle (M), and small surface proteins (S).

Fig. 2. Study of viral DNA replication and protein synthesis in HepG2 cells. (a) Dot blot kinetic of viral DNA in the supernatants over 7 days following infection of HepG2 cells with HBV* and HBV or seeding of HepG2.2.15 cells. (b) Infectivity of HBV* for HepG2 cells; prior incubation of HBV with PDLCs was performed for 4 or 24 h (lanes 2 and 3). Alternatively HBV-treated PDLCs or primary duck kidney cells were transferred into the HepG2 culture (lanes 5 and 6); lane 1, HepG2.2.15 cells; RC, relaxed circular, and SS,

single-stranded, DNAs of HBV; M, viral marker DNA. (c) Kinetic study of HBs for one week; here, day 1 represents viral input. All data points represent the mean of 3 calculations. (d) Analysis of the production of viral surface (left) or core (21.5 kD) proteins (right) by Western blot one week after infection with HBV* or HBV; mock*- and mock-treated inputs refer to the supernatants of HepG2 cells similarly passaged over PDLCs or not; P, PDLCs after removal of HBV*.

Fig. 1. Maenz & Bruns

Fig. 2. Maenz & Bruns