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Animal models for hepatitis B: does the supply meet the demand?

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Worldwide, over 257 million people are persistently infected with hepatitis B virus (HBV), which can lead to a broad spectrum of disease outcomes, including cirrhosis and/or hepatocellular carcinoma (HCC). HBV can be effectively prevented with a prophylactic vaccine, and currently approved antiviral therapy can suppress viremia but hardly cure the underlying infection. New insights into the viral life cycle and HBV's interactions with the host cell have re-ignited efforts to devise improved antiviral therapies with the ambitious goal of completely eradicating HBV or permanently inactivating the virus in patients. Systematic testing of approaches to cure HBV has been hampered by the scarcity of animal models faithfully recapitulating infection and the clinical features associated with chronic hepatitis B.

HBV is a partially double-stranded DNA virus of the *Hepadnaviridae* family, genus *Orthohepadnavirus.* Although viruses genetically similar to HBV have been identified in a variety of species [1], the etiologic agent of HBV in humans has a remarkably narrow tissue and host range, limited to hepatocytes in humans and chimpanzees [2, 3]. The mechanistic

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basis for this highly restricted tropism has not been fully deciphered, and consequently it has proven difficult to establish the entire viral life cycle in traditionally non-permissive species.

Do we still need (more) animal models for hepatitis B?

Chronic hepatitis B is a multifarious disease resulting from the intricate interplay between HBV, hepatocytes, non-parenchymal cells and the immune system. Tremendous progress has been made towards modeling HBV infection in increasingly complex cell culture and tissue organoid platforms [4], but none of them adequately capture the complexity of the disease. The highly restricted species tropism of HBV, which is limited to chimpanzees and humans [2, 3], has made *in vivo* study of this virus notoriously difficult. This has become even more challenging with the NIH moratorium on research in chimpanzees, underscoring the need for alternative small animal models suitable for studying HBV pathogenesis. Considerable progress has been made in understanding the basic mechanisms of HBV replication, but the precise host factors needed for completion of the virus's replication cycle remain to be fully elucidated. We have insufficient insights into how HBV is sensed by the host's innate and adaptive immune responses and how host defenses may contribute to the markedly severe pathogenesis caused by HBV, which is widely considered a non-cytopathic virus. Although the HBV replication cycle can be mimicked by other genetically related viruses, such as woodchuck hepatitis virus (WHV) in WHV-infected woodchucks [5], the genetic diversity of woodchucks and lack of immunological tools precludes their use as a practical model. Nonetheless, studies using these surrogate models have been very informative as they have provided important pathogenic insights and have been invaluable testing antiviral treatment approaches against HBV infection [6]. Without an established small animal model that supports the entire life cycle of HBV and recapitulates human disease phenotypes, our ability to fully understand HBV interactions with infected cell and the immune system and to develop effective antiviral treatment against these dreadful infections, remains limited. Immunocompetent animal models may also lend themselves to evaluating improved HBV prophylactic (e.g. single dose) and therapeutic vaccines.

Distinct (new) biomarkers to track HBV infection in peripheral circulation can be further evaluated in animal models. These include but are not limited to circulating HBV RNA (pgRNA), quantitative HBsAg, HBeAg and HBcrAg as well as anti-HBs and -HBc antibodies. Establishing profiles of such markers that can unambiguously discern between e.g. viral suppression and a (functional) cure would be invaluable for interpreting clinical trial data.

Given the paucity of patient liver biopsies, which are rarely taken during clinical trials due to safety concerns, animal models also play a key role in translational research as they permit to assess the impact of novel antiviral therapies on virological (e.g. intrahepatic replication intermediated) and host parameters (e.g. immune cell infiltration, transcriptional, proteomic of metabolomic changes) within the infected liver. Previous work in humans suggested that intrahepatic cccDNA levels may be studied through evaluating circulating blood cell free DNA or in circulating cells that have been released from the liver [7]. Studies in animal models would undoubtedly help to determine how well such peripheral surrogates correlate with intrahepatic replication levels.

What are desired features of (an) animal model(s) for hepatitis B?

Optimally, any animal model for hepatitis B should closely mimic as many of the relevant clinical features observed in patients as possible. HBV animal models should be readily susceptible to all viral genotypes, support formation of covalently closed circular DNA (cccDNA) and result in persistent viremia, in the majority of exposed animals. The model should be fully immune-competent in order to mechanistically dissect correlates of protective immunity, persistence and immune-mediated pathogenesis. Since in humans decades can pass between the acute phase of infection and development of severe liver pathologies, it is impractical, and perhaps even unrealistic, to aim for experimental models to follow this same extended progression timeline. To overcome this challenge, it would be desirable if the model(s) was/were amenable to genetic manipulations that possibly allowed for accelerated and/or exacerbated development of clinically relevant symptoms. From a practical perspective, an animal model for hepatitis B should be highly reproducible, easy to propagate, high throughput and affordable to produce.

Multipronged approaches towards creating animals for hepatitis B infection and immunity

Currently, no single animal model exists for HBV that combines all of the desired features outlined above. However, there are several models that at least recapitulate aspects of the viral life cycle (recently reviewed detail [6], see also table 1) and have served as valuable models for evaluating various antivirals in preclinical studies.

Rodent models would undoubtedly be most convenient due to their low-cost, rapid propagation and the prevalence of experimental tools for their genetic manipulation. However, mice and rats are resistant to HBV infection. The discovery of the human sodium taurocholate co-transporting peptide (hNTCP, or SLC10A) as the receptor for HBV and hepatitis delta virus[3] raised hopes that murine models with inbred susceptibility to HBV infection could be generated. However, while HBV can enter hNTCP-expressing hepatocytes in mice, blocks in subsequent steps of the viral life cycle hinder further progression of infection [8]. *In vitro* evidence suggests that cccDNA formation is supported in certain murine hepatoma cell clones [9], which is consistent with the fact that the minimal factors required for this step[10] are highly conserved across species. These data indicate that other steps in the HBV infection cycle, likely prior to cccDNA formation, may not be supported. Systematic dissection of the blocks in interspecies transmission holds the key towards establishing an HBV-susceptible mouse model.

Transgenic or viral (e.g. AAV or adenovirus) vector-mediated expression of the HBV genome has proven effective in bypassing some of these restrictions. Although such models have shown some utility in testing therapeutics and studying aspects of infection [11, 12], such as HBV-specific immune responses in rodents, one should keep in mind that an actual infection is not established as the virus cannot spread: the infectious virions produced and released from cells harboring the viral genome are unable to enter naive cells.

Humanized xenotransplantation models represent a valuable complementary system. Robust engraftment of human hepatocytes into various immunodeficient liver injury models has been reported (reviewed in [13]). Progress has been made in engrafting *in vivo* expanded primary human hepatocytes [14, 15] and stem cell derived hepatocytes [16] which will aid in increasing throughput, opens the possibility of manipulating genetically human hepatocytes and reduces donor-to-donor variability. Although, one shortcoming of the current models is that other non-parenchymal, hepatic cell populations critical for cellular crosstalk are not humanized. Regardless, the resultant human liver chimeric mice are susceptible to HBV [17] and several other human hepatotropic pathogens [13] and are a valuable tool for testing antiviral therapeutics [18–20]. However, their highly immunocompromised nature limits their utility for evaluating any antiviral approaches relying on immune-stimulation and/or -modulation. Protocols are being continuously refined to achieve robust co-engraftment of hepatocytes and components of a human immune system (HIS). Such dually engrafted mice can support HBV infection, and studies have shown that viral infection triggers activation of the engrafted HIS [21], particularly natural killer (NK) cells [22] and M2 macrophages [23], and leads to some virally induced histopathology [23].

It should be noted, however, that sourcing donor-matched hepatocytes, non-parenchymal cells and hematopoietic stem cells (HSCs) remains challenging at present. While stem-cell derived cells may provide a possible solution, currently available protocols for differentiating ES or iPS cells into the hepatic lineage and HSC do not yield cells that can be engrafted reproducibly and robustly into commonly used xenorecipient strains.

Future refinements will focus on improving the limited functionality of the engrafted HIS. Numerous strategies to do this have been proposed (reviewed in [24, 25]) as several human cell lineages remain under-represented, in part due to the orthologs of non-redundant cytokines exhibiting limited biological species cross-reactivity. It was previously shown that selective expansion of under-represented cell types, such as dendritic cells, NK cells and granulocytes, leads to immune responses to the live attenuated yellow fever virus (YFV) vaccine more akin to those observed in YFV vaccinees [26]. Promisingly, expression of murine thymic stromal lymphopoietin (TSLP) can restore lymph node development in immunocompromised mouse strains used for xenotransplantation and drastically augments human immunity to model antigens [27]. Additionally, the development of functional adaptive immune responses has been limited by the lack of HLA gene expression. Expressing a human MHC class I allele has multiple benefits allowing for more faithful development of CD4+ and CD8+ T cells in the thymus, enabling recognition of (viral) antigens in peripheral tissues by human T cells and facilitating the tracking of antigen-specific CD8+ T cells with MHC multimers, as previously shown for EBV, dengue virus and YFV [28–30]. Co-engraftment of improved xenorecipient strains with additional hematopoietic stem cell donor-matched human tissues, such as liver, thymus and/or lymph nodes, could also significantly augment the immune response. Such co-engraftments could enhance T and B cell selection, intra-hepatic T cell priming [31] and liver-mediated secretion of key human immune components [32]. Finally, engraftment of second-generation humanized mice with a human-like microbiome represents another valuable approach to enhance immunity, as previously suggested [33].

Non-human primates (NHPs) would likely provide the strongest immune data for translation into clinical trials, given their similar immune systems and the wealth of reagents available. Work in macaques has shown that expression of hNTCP in the liver results in HBV viremia, albeit transiently and at low levels [34]. Ideally, additional steps towards making an improved NHP model for HBV include creation of transgenic rhesus macaques susceptible to HBV and alteration of HBV PreSI to accommodate binding and entry using the macaque NTCP receptor.

The bottom line is that the scarcity of accessible immunocompetent models of chronic HBV infection is a critical roadblock in the journey to find a cure for HBV. Currently available models are either immunodeficient, do not truly model infection or use surrogate viruses in rare species. Each of these weaknesses create distinct challenges in translating pre-clinical results to humans. Ideally, immunocompetent chronic infection models would be established in both mice and NHPs. Developing such models would allow for more relevant studies in species for which immunologic reagents and protocols are already available for a broad range of targets and pharmacodynamic markers. Furthermore, studies could initially be run at a lower cost and higher throughput in mice before being subsequently confirmed in the costlier, but likely more relevant, primate system.

A call to action!

To reduce the disease burden caused by HBV, it will undoubtedly be key to continue educating the public to overcome the stigma associated with this disease, to systematically identify HBV carriers and to promote widespread use of the available prophylactic HBV vaccine. However, these measures fall short in helping the large number of patients already persistently infected. There is irrefutable, albeit sporadic, evidence that chronic hepatitis B can be cured, providing hope for patients and motivation for scientists and clinicians alike that novel therapeutics can result in a (functional) cure. This arguably ambitious goal will take an all hands on deck approach to develop critical reagents such as HBV animal models in line with the following key principles: 1. To be truly transformative, any animal model development efforts need to focus on the actual needs of potential end users and meet the highest standards of rigor and reproducibility; 2. Whenever possible, necessary tools and reagents ought to be made accessible through public repositories such as BEI, a repository developed by NIAID (https://www.beiresources.org); 3. To support multipronged approaches to developing a HBV animal model, robust, long-term investments – from both the private and public sector – remain crucial; 4. The evaluation process for publicly funded research must be insulated from politics and allowed to impartially identify promising research proposals that are ethically sound as well as scientifically and technically rigorous. Installing e.g. a NIH Human Fetal Tissue Ethics Advisory Board that has the predetermined objective of blocking the use of critically needed human fetal tissue is impeding promising biomedical research that has the potential to save lives and reduce human suffering caused by hepatitis B and many other diseases; 5. Finally, efficient information exchange not only within academia as well as with industry will be important to keep all parties abreast of key developments. To this end, the International Coalition to Eliminate HBV (ICE-HBV) workshop and other outlets serve as a viable and increasingly important platform.

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Table 1:

Comparison of different animal model for HBV

		HBV life-cycle supported?							
species	**model**	**entry**	**replication**	**assembly/ release**	**Immune status**	**throughput**	**costs**	**utility**	**caveats**
mouse	1.3x HBV transgenic	no	yes*	yes	immunocompetent but tolerized to HBV	high	low	immune-responses, some pathogenesis	not an authentic infection model
	AAV-/AdV-HBV	no	yes*	yes	immunocompetent	high	low	immune responses, immunomodulators	not an authentic infection model
	hNTCP transgenic/ knock-in	yes	no	no	immunocompetent	high	low	entry inhibitors?	only entry
	human liver chimeric	yes	yes**	yes	immunodeficient	medium	medium	testing of DAAs and some HTAs	immunodeficient
	dually engrafted human liver/ immune system mice	yes	yes**	yes	immunocompetent	medium	medium	immune-responses, some pathogenesis, immunomodulators	heterogeneity, limited immune functionality
Rhesus macaque	AAV-hNTCP	yes	yes**	yes	immunocompetent	low	high	immune-responses	only low level, transient viremia
Tree shrews	HBV	yes	yes**	yes	immunocompetent	low	medium	TBD	weak, short-lived viremia with limited viral replication
Woolly monkey	WMHBV	yes	yes**	yes	immunocompetent	low	n/a	n/a	surrogate virus, endangered species, not accessible
Spider monkeys	WMHBV	yes	yes**	yes	immunocompetent	low	n/a	n/a	surrogate virus, endangered species, not routinely available for research
Squirrel monkey	HBV/WMHBV	yes	yes**	yes	immunocompetent	low	high	n/a	surrogate virus, only low level, transient WMHBV viremia, limited availability of tools
woodchuck	WHBV	yes	yes**	yes	immunocompetent	low	high	vaccine development, assessing immunologic aspects of the disease, and evaluating antiviral therapies	surrogate virus, difficult to access, limited availability of tools
ducks	DHBV	yes	yes**	yes	immunocompetent	high	high	vaccine development, assessing immunologic aspects of the disease, and evaluating antiviral therapies	surrogate virus, limited availability of tools

*does not occur exactly as de novo HBV replication.

**denotes clear evidence for cccDNA formation

AdV, adenovirus; AAV, adeno associated virus; cccDNA, covalently closed circular DNA; DAA, directly acting antiviral; DHBV, duck HBV; HBV, hepatitis B virus; HTA, host targeting antiviral; WMHBV, woolly monkey HBV; WHBV, woodchuck HBV