

# Hepatitis B and Delta Virus: Advances on Studies about Interactions between the Two Viruses and the Infected Hepatocyte

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## Abstract

The mechanisms determining persistence of hepatitis B virus (HBV) infection and long-term pathogenesis of HBV-associated liver disease appear to be multifactorial. Although viral replication can be efficiently suppressed by the antiviral treatments currently available, viral clearance is generally not achieved since HBV has developed unique replication strategies, enabling persistence of its genome within the infected hepatocytes. Moreover, no direct antiviral therapy exists for the more than 15 million people worldwide that are also coinfecting with the hepatitis delta virus (HDV), a defective virus that needs the HBV envelope proteins for propagation. The limited availability of robust HBV and HDV infection systems has hindered the understanding of the complex network of virus-virus and virus-host interactions that are established in the course of infection and slowed down progress in drug development. Since chronic HBV/HDV coinfection leads to the most severe form of chronic viral hepatitis, elucidation of the molecular mechanisms regulating virus-host interplay and pathogenesis are urgently needed. This article summarizes the current knowledge regarding the interactions among HBV, HDV, and the infected target cell and discusses the dependence of HDV on HBV activity and possible future therapeutic approaches.

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**Keywords:** HBV; HDV; HBsAg; HDAg; Immune system; Hepatocytes; Virus interactions.

**Abbreviations:** ALT, alanine aminotransferase; CHB, chronic HBV; ER, endoplasmic reticulum; HBV, hepatitis B virus; HBxAg, HBV X antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDV, hepatitis delta virus; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; HDAg, hepatitis delta antigen; ISGs, interferon stimulated genes; NF- $\kappa$ B, nuclear factor kappa B; NK, natural killer; NTCP, sodium taurocholate cotransporting polypeptide; NUCs, nucleoside/nucleotide analogues; pgRNA, pregenomic RNA; ROS, reactive oxygen species; SCID; severe combined immunodeficiency; SRE, serum response element; STAT, signal transducer and activator of transcription; TNF- $\alpha$ , tumor necrosis factor alpha; USB mice, UPA/SCID/beige mice; UPA, urokinase-type plasminogen activator; WHO, World Health Organization.

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## Hepatitis B virus

About 2 billion people worldwide have been in contact with the hepatitis B virus (HBV), and according to the latest estimation of the World Health Organization (WHO), 240 million are chronically infected with HBV. Every year around 780,000 people die due to the consequences of HBV infection.<sup>1</sup> HBV belongs to the hepadnaviridae family and is a human-specific virus with a unique genome structure and replication cycle.<sup>2</sup> Ten HBV genotypes have been described to date. Genotype A is prevalent in Africa and Northwestern Europe, while genotypes B and C are mainly found in Asia, Australia, and New Zealand. Genotype D is predominant in Mediterranean countries, the Middle East, Central Asia, and India. Genotype E is restricted to West Africa, genotype F and H to Mexico and South America, and genotype G to the United States and France. In the United States, genotypes A, B, C, D, and G have been found.<sup>3</sup> Moreover, another genotype (I), was isolated in Vietnam<sup>4</sup> and Laos<sup>5</sup> in 2008, and genotype J was identified in Japan in 2009.<sup>6</sup> However, due to the recent increase in the number of travelers and migrants, the original geographic distribution of the HBV genotypes cannot be applied as strictly anymore, and significant changes in the distribution will likely occur.

The circular HBV genome is approximately 3,200 base pairs (bp) long and exists in infecting virions (Dane particles) as a relaxed circular, partially double-stranded deoxyribonucleic acid (rcDNA). It is covalently linked to the terminal protein of the viral polymerase inside the nucleocapsid, which is formed by the hepatitis B core antigen (HBcAg).<sup>7</sup> The viral envelope encloses the nucleocapsid and consists of a lipid membrane and the hepatitis B surface antigens (HBsAg), which exist in three different forms: small, medium, and large.<sup>8</sup> The sodium taurocholate cotransporting polypeptide (NTCP), a multiple transmembrane transporter localized to the basolateral membrane of highly differentiated primary hepatocytes, was identified as the bona fide receptor that permitted HBV and HDV entry into the hepatocytes.<sup>9</sup> The entry pathway following HBV binding to the cell membrane has not been fully elucidated, but experimental evidence indicated that HBV was involved in an endocytosis process, followed by the release of the nucleocapsid from endocytic vesicles. Previous studies also indicated that the viral capsids were transported via microtubules to the nuclear periphery,<sup>10</sup> where capsid accumulation would facilitate interactions with nuclear transport receptors and adaptor proteins of the nuclear pore complex.<sup>11</sup> Within the nuclear baskets,

disintegration of the capsids shall permit the release of both core capsid subunits and of the viral DNA polymerase complexes into the nucleoplasm.<sup>12</sup> Although the mechanisms driving the formation of the covalently closed circular form (cccDNA) remain largely unknown, the establishment of productive HBV infection requires the removal of the covalently attached viral polymerase and completion of the positive-strand by the cellular replicative machinery to form the supercoiled cccDNA molecule. This is then incorporated into the host chromatin and serves as the template of viral transcription and replication.<sup>13,14</sup> Recent studies provided evidence that HBV used cellular DNA repair enzymes to remove the P protein and initiate cccDNA biogenesis.<sup>13,15,16</sup> Using the cellular transcriptional machinery, the cccDNA serves as a stable template for the transcription of all viral ribonucleic acids (RNAs) necessary for protein production and replication of progeny viruses, which takes place in the cytoplasm after reverse transcription of an over-length pregenomic RNA (pgRNA). The stability of the cccDNA in hepatocyte nuclei plays a crucial role in the persistence of HBV infection.<sup>17</sup> Other viral proteins, which are generated within the replication cycle of HBV, are the HBeAg and the regulatory X protein. HBeAg is a nonstructural protein that is excreted from the infected hepatocyte into the blood and appears to act as a decoy for the immune system.<sup>2</sup> The function of the HBV X antigen (HBxAg) is not completely understood, but it has been shown to be essential for cccDNA transcription *in vivo*.<sup>18</sup>

The transmission of HBV occurs parenterally through infected blood or body fluids. In high endemic areas, HBV infection originates from transmission of an infected mother to her child during birth (perinatal transmission), while in low prevalence countries HBV is mainly transmitted through unprotected sexual contact and needle sharing among drug users.<sup>19</sup> After an incubation time of 1 to 6 months, 2/3 of cases will have an acute HBV infection without symptoms (asymptomatic course), and 1/3 of patients will develop unspecific symptoms, such as fatigue, weight-loss, anorexia, and nausea, and progress to disease with liver specific symptoms (jaundice and liver failure). Approximately 90% of acute infections in adults resolve spontaneously with the development of long-lasting immunity,<sup>20</sup> while the remaining 10% develop a chronic HBV infection that over years is frequently associated with liver inflammation, leading to cirrhosis and increases in the incidence of hepatocellular carcinoma (HCC).<sup>21</sup> Moreover, 90% of children infected before 1 year of age develop a chronic HBV infection.<sup>22</sup> Approved therapeutic agents that decrease the morbidity and mortality of a chronic HBV infection are pegylated interferon- $\alpha$  and nucleoside/nucleotide analogues (NUCs). Although a seroconversion of HBeAg and serum HBV DNA levels below the lower limit of detection might be achieved with current therapeutics, a loss of HBsAg is rarely observed, and the complete eradication of the infection seems to be not possible.

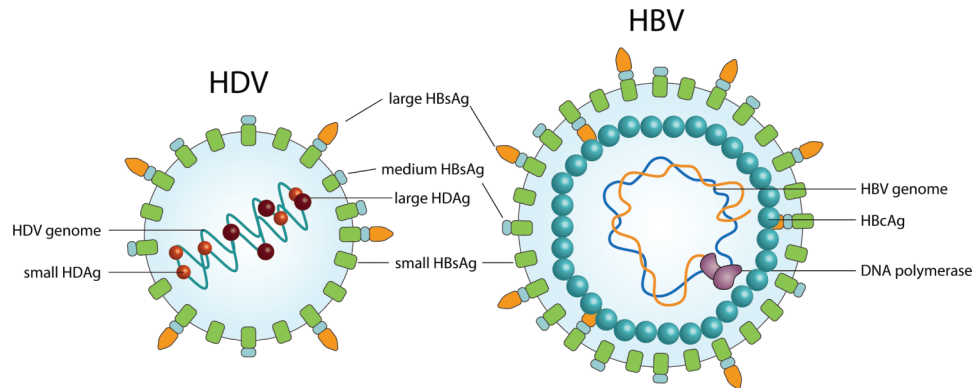
Interferon- $\alpha$  acts both by modulating the immune system (through stimulation of interferon stimulated genes (ISGs) and modulation of natural killer (NK) cells)<sup>23-25</sup> and by inducing direct antiviral effects, including epigenetic suppression of cccDNA transcription.<sup>26</sup> NUCs (e.g. lamivudine, adefovir, and entecavir) inhibit the HBV polymerase<sup>27</sup> but do not influence cccDNA-driven RNA transcription. Consequently, subgenomic RNAs coding for the envelope proteins, which are mostly secreted as subviral particles, are still produced, explaining why significant HBsAg reduction and seroconversion are rarely achieved with NUC-based

treatments.<sup>28</sup> Thus, novel therapies considering alternative antiviral targets and aiming to achieve a functional HBV cure are urgently needed. Among the new candidate drugs targeting steps of the viral life cycle distinct from HBV replication, Myrcludex-B, which is a myristoylated synthetic peptide binding NTCP and inhibiting viral attachment and entry, was shown to block efficiently the establishment of HBV infection and to hinder intrahepatic cccDNA accumulation in the spreading phase of infection both *in vitro* and *in vivo*, using humanized mice.<sup>29,30</sup> The use of drugs efficiently protecting the hepatocytes from reinfection may represent an interesting therapeutic approach, since it could contribute to lower intrahepatic viral loads when used in combination with agents that aim to reduce not only HBV replication but to promote cccDNA destabilization and/or restoration of immune responses.

### Hepatitis Delta virus

The hepatitis delta virus (HDV) was discovered in 1977 by Marcus Rizzetto.<sup>31</sup> The outbreak, which started in Italy, has been brought under control in industrialized countries during the past 20 years.<sup>32,33</sup> Nevertheless, still more than 15 million people worldwide are estimated to be chronically infected with HDV,<sup>34</sup> especially in developing continents like Asia and Africa where HDV infections remain a major health problem.<sup>35</sup> Three major genotypes with highly variable sequences, which were further subdivided into eight HDV clades, have been reported.<sup>36</sup> However, this classification was lately revised to group HDV subtypes into eight genotypes.<sup>37</sup> With the exception of genotype 1, which is the most frequent one and diffused worldwide, the other genotypes appear to be restricted to certain geographical areas: HDV-2 and 4 are mostly found in the Far East and Russia; HDV-3, which has been associated with the most severe form of chronic hepatitis, is mostly observed in the northern parts of South America in the Amazonian region; while genotypes 5, 6, 7, and 8 are generally found in Africa or African migrants.<sup>38</sup>

The hepatitis delta virion is the smallest RNA pathogen known to interact with a human host and to cause substantial global morbidity and mortality. The inner nucleocapsid of the virus contains a 1,679 nucleotide long, single-stranded, circular RNA (genomic HDV RNA) and around 200 molecules of hepatitis delta antigen (HDAg), which is the only known protein encoded by the HDV RNA. Within the hepatocyte, replication leads to the accumulation of three HDV RNAs: the circular genomic RNA, the antigenomic RNA, and a smaller linear mRNA, which is the template for the translation of HDAg. HDV uses a so-called rolling cycle amplification mechanism and the host RNA polymerase II to transform the genomic HDV RNA into its exact complementary form, the antigenomic HDV RNA and then into new viral genomes.<sup>39,40</sup> A unique open reading frame on the antigenomic HDV RNA leads to the synthesis of the HDAg, which occurs in two different forms: small HDAg and large HDAg.<sup>41</sup> The small HDAg (24 kDa) is important for virus replication, whereas the large form (27 kDa) inhibits replication and leads to virion assembly.<sup>42,43</sup> HDV is a defective virus, whose genome is surrounded by three HBV envelope proteins and host lipids (Fig. 1).<sup>44</sup> HBV plays an essential role as a helper virus for HDV, since its envelope proteins are stringently necessary for HDV propagation.<sup>45</sup> Therefore, the release of hepatitis delta virions from the infected hepatocytes can only occur if the



**Fig. 1. HBV as a helper virus for HDV.** Hepatitis B virus (HBV) shares its small, medium, and large hepatitis B surface antigens (HBsAg) with hepatitis D virus (HDV).

cells are coinfecting with HBV or when HDV super-infection occurs in individuals already infected with HBV.

By sharing the same viral envelope (HBV viral proteins), HDV is also transmitted parenterally through infected blood or body fluids. Notably, HBV/HDV coinfections often cause more severe symptoms than HBV mono-infections. An acute coinfection emerges after an incubation time of 3 to 7 weeks and can either take an asymptomatic course, show several non-specific symptoms (like fatigue, lethargy, anorexia, and nausea), or result in acute liver failure.<sup>46</sup> In the setting of an HDV super-infection, up to 80% of the patients show a chronic course of disease, which is associated with liver inflammation, fibrosis, and decompensated liver cirrhosis. It is known that a super-infection with HDV and high HDV viremia levels increase the risk for a rapid progression to cirrhosis and HCC in comparison to an infection with HBV alone.<sup>39,47</sup> In contrast, an HBV/HDV coinfection mostly occurs as an acute, self-limited infection and leads in less than 5% to a chronic manifestation.<sup>48</sup> Since HBV vaccine is directed against the same envelope proteins that are used by HDV, vaccination against HBV also protects against HDV infections in individuals who are not already HBV-infected. Unfortunately, therapy options for HDV are very limited. There is no HDV-specific treatment, and currently the only approved drug is pegylated interferon- $\alpha$ , which leads to unsatisfactory outcomes in HDV infected individuals. Different clinical trials revealed that sustained virological responses were achieved only in approximately 30% of HDV infected patients at the end of follow-up,<sup>49–51</sup> demonstrating recrudescence of HDV after treatment discontinuation. Due to its simplicity in structure and lack of producing its own polymerase, HDV offers much fewer targets than other viruses and common HBV therapeutics cannot directly target HDV infection. Unfortunately, only a minority of patients treated with interferon clear HDV infection. Since HDV does not use a viral polymerase for replication, potent NUCs efficiently suppressing HBV reverse transcription do not show beneficial effects against HDV infection.<sup>52–54</sup> Also ribavirin, which in combination with interferon was successfully used in chronic hepatitis C virus (HCV) infections and was reported to have activity against HDV based upon *in vitro* studies,<sup>55</sup> appeared ineffective for the treatment of HDV infected patients.<sup>56</sup> Very few new therapeutic approaches exist. Myrcludex-B is a promising entry inhibitor not only in HBV but also in HDV infections.<sup>57</sup> An alternative to interferon- $\alpha$  might be treatment with interferon- $\lambda$ , which appears to cause fewer side

effects since its specific cellular receptor is restricted to cells of epithelial origin.<sup>58</sup> A previous study revealed that prenylation of the large HDAg is essential for virus assembly and release and that prenylation inhibitors are able to decrease HDV RNA levels *in vivo*.<sup>59</sup> Thus, the development of efficient therapeutic approaches, which directly target HDV replication, is urgently needed.

#### Interactions between HBV and HDV: *in vitro*, *in vivo*, and clinical studies

Since HDV requires the envelope proteins of HBV (HBsAg) for its assembly and release, a productive HDV infection, leading to the release of progeny viruses, exclusively occurs in the presence of HBV. It is thus plausible that both viruses have to interact with each other at different stages of their replication cycles. However, knowledge about the exact interplay between both viruses and to what extent HDV may influence HBV life cycle remains limited.

One of the first studies to address these questions was published in 1989, when direct inoculation of HDV genomes into the liver of an HBV infected chimpanzee showed transient HBV reduction during the acute phase of HDV infection.<sup>60</sup> In 1990, Wu *et al.* cotransfected a human hepatoma cell line with plasmids coding for HBV and HDV genomes and found that HBV RNA transcriptional levels were dramatically suppressed in comparison to cells that were transfected with an HBV plasmid alone. Since a similar suppression of HBV RNAs (9- to 17-fold) and released HBV virions (9-fold) was also detected when these cells were cotransfected with an HBV plasmid and a plasmid expressing only the HDAg, Wu *et al.* concluded that the HBV RNA suppression must be mediated by the HDAg.<sup>61</sup> Interestingly, the levels of genomic and antigenomic HDV RNA produced *in vitro* were not affected by the presence of HBV, suggesting that HBV had no influence on HDV replication.<sup>61</sup>

Studies performed in mice harboring human livers (urokinase-type plasminogen activator (UPA)/severe combined immune deficiency (SCID)/beige mice (USB mice)) that were first stably infected with HBV and then super-infected with HDV, demonstrated a 0.6 log reduction of HBV viremia. In addition, the development of HBV viremia and intrahepatic cccDNA loads in humanized mice appeared to be delayed in the setting of HBV/HDV coinfection and in comparison to virological parameters obtained in HBV mono-infected

animals. Taken together, these findings indicated that HDV may hinder HBV replication.<sup>57</sup>

The observation that HDV can suppress HBV in the setting of a co- or super-infection was also made in several retrospective patient studies.<sup>62–66</sup> In 1991, Sagnelli *et al.* investigated 171 patients chronically infected with HBV, who were HDAg-positive ( $n=31$ ), HDAg-negative but antiHDAg-positive ( $n=54$ ), or did not show any signs of present or past HDV infection ( $n=86$ ). Intrahepatic HBcAg was detected in 50% of the HBV monoinfected cohort, while only 6% of the HDAg-positive and 13% of the antiHDAg-positive patients had HBcAg-positive hepatocytes.<sup>66</sup> Nevertheless, Colombo *et al.* described that hepatic inflammation was rather related to HBcAg levels in the liver of HBV infected individuals. Since such inflammation appeared to be independent of HDV super-infection, the study suggested that the levels of HBV replication, rather than HDV activity, might play a predominant role in causing liver damage among HBV/HDV infected individuals.<sup>63</sup> However, controversial data exist about the ability of HDV to suppress HCV in the setting of HBV/HCV/HDV triple-infection. For instance, in a recent clinical study, Jardi *et al.* analyzed HBV/HDV coinfecting, HBV/HCV/HDV triple-infected, as well as HBV and HCV monoinfected individuals and found that HDV appeared to suppress both HBV and HCV viremia, though HCV replication was reduced to a greater extent than HBV.<sup>67</sup> Moreover, Heidrich *et al.* investigated virological patterns in a cohort of 258 HDV infected patients from Central Europe and showed that HDV infection was associated with suppression of both HBV and HCV replication.<sup>68</sup> In contrast, HBV DNA and HCV RNA levels did not seem to influence HDV replication in that patient cohort, and mean HBsAg levels did not significantly differ between HBV monoinfected and HBV/HDV coinfecting patients.<sup>68</sup> While Eyster *et al.* and Mathurin *et al.* also supported the observation that HDV predominated HCV in triple-infected patients by failing to detect serum HCV RNA and markers of HBV replication in most of the patients,<sup>69,70</sup> Liaw *et al.* showed that HCV is the dominant virus in triple infected patients in Asia.<sup>71</sup> Since these studies were conducted in different patient populations, the discrepancies suggested that patient polymorphisms, viral genotype varieties, as well as environmental factors add complexity and have to be taken into account, since all these factors may strongly influence virus-virus interactions and the clinical outcome of the infection. Although infection with HDV is frequently associated with a more severe disease course compared to other hepatitis virus infections, different HDV genotypes have been associated with different clinical manifestations. For example, HDV genotype 3 is considered to cause a more fulminant hepatitis than genotypes 2 and 4, while patients infected with HDV genotype 1 can develop a wide range of severity.<sup>72</sup> Clinical<sup>73,74</sup> and *in vitro* observations<sup>75</sup> demonstrated that various combinations with different HBV genotypes are possible and that some HBV strains seem to favor HDV assembly and improve HDV infectivity;<sup>75</sup> factors that might also influence clinical manifestations. In contrast, Shih *et al.* did not find a positive correlation between replication and assembly capacities of HDV in relation to distinct HBV genotypes, thus questioning the impact of different HBV genotypes on clinical outcomes of HDV infections.<sup>76</sup>

To investigate the interplay of HBV and HDV during the course of coinfection, an interesting longitudinal study was published by Schaper *et al.* in 2010, where HBV and HDV replicative activities were evaluated for up to 8 years in 25

chronically HBV/HDV coinfecting patients.<sup>77</sup> Interestingly, seven different replication profiles were observed in these patients. 20% of the coinfecting individuals showed a persistent activity of HDV in the absence of HBV activity; 12% demonstrated a persistent activity of both viruses, while another 12% showed persistent HBV activity in the absence of HDV replication. The remaining 56% of patients showed a fluctuating activity of both viruses (24%) or of one of the two viruses (32%). HDV was dominant in most of the patients observed (60%), but a predominance of HBV (16%) or none of the two viruses was also determined in a remarkable number of coinfecting individuals (24%).<sup>77</sup>

What we should learn from the results obtained from these different cross-sectional and longitudinal clinical studies is that HDV seemed to be able to suppress HBV and HCV at certain time points in the course of concomitant infection. Since multiple virological and host-related events (i.e. viral genotypes, immune state, and environmental factors) may affect viremia levels, HDV cannot generally and necessarily be considered the predominant virus in all HBV/HDV coinfecting patients based on a single determination.<sup>78</sup>

### Interactions between HBV and HDV: role of the HBsAg

During the replication cycle of HDV, the surface proteins of HBV specifically interact with the HDAg at the endoplasmic reticulum (ER) of the infected cells and, hence, are essential for HDV assembly.<sup>79</sup> The detection of HBsAg in serum is not only a fundamental diagnostic marker of HBV infection, but it may also be a promising prognostic marker during the natural history of chronic HBV infection and antiviral therapy.<sup>80</sup> The natural history of chronic HBV (CHB), in general, is regarded to consist of four phases: immune tolerant phase, HBeAg-positive chronic hepatitis (immune clearance), immune control (with low or nonreplicative HBV), and HBeAg negative hepatitis (immune escape). These phases have been identified on the basis of specific biochemical, serological, and virological characteristics, including serum alanine aminotransferase (ALT) levels, HBeAg status, viremia, and HBsAg levels.<sup>81</sup> In the acute and early stages of CHB mono-infection (immune tolerant phase), HBsAg often correlates with serum HBV DNA levels in infected patients.<sup>82</sup> Moreover, HBsAg levels can vary among HBV genotypes as well as fluctuate over the years.<sup>83,84</sup> Jaroszewicz *et al.* investigated 226 HBV mono-infected individuals at different phases of chronic infection and detected no or weak correlations between HBsAg and viremia, indicating that HBsAg production and HBV DNA replication can differ at later time points of CHB (i.e. during immune clearance and control).<sup>80</sup> This could be due to the accumulation of HBV DNA integrations that may provide a separate template for HBsAg production, the emergence of variants (where HBeAg-negative variants are the most frequently found), or immune-mediated factors, including cytokines, which affect cccDNA transcription.<sup>78</sup> On the other hand, Volz *et al.* found that serum HBsAg levels were significantly lower in HBeAg-negative individuals and that lower intrahepatic cccDNA levels correlated with lower HBsAg concentrations in serum.<sup>83</sup>

Cross-sectional studies showed in HBV/HDV coinfections that HBsAg levels were usually high, despite lower levels of HBV viremia.<sup>85</sup> Moreover, Shih *et al.* found a positive correlation between HDV productivity and expression levels of HBsAg but not with HBV DNA levels or HBV genotypes.<sup>76</sup> In contrast, a longitudinal study performed by Schaper *et al.*



demonstrated that levels of circulating HBsAg in HBV/HDV coinfecting patients showed significant fluctuating profiles, suggesting that HDV may be directly responsible for the HBsAg flares.<sup>77</sup> However, contrary to the earlier cross-sectional studies, this study failed to detect a positive correlation between HBsAg levels and amounts of HDV RNA.<sup>77</sup> It should be noted that these clinical observations are not supported by experimental evidence, and a complex interplay of virological and host factors may be involved. At present, it can be only hypothesized that HBV viremia and HBsAg fluctuations reflect in part a specific suppression of the HBV replication pathway, which could occur, for instance, by sequestering the envelope proteins needed for HBV release or by differentially affecting distinct HBV promoters. Future studies using well controlled experimental systems will be needed to address these hypotheses.

Infectious HDV particles can enter and replicate within a hepatocyte in the absence of HBV, since these steps only need the presence of host factors and enzymes.<sup>86</sup> The coexistence of both viruses, and in particular the HBV envelope proteins, in the same hepatocyte is necessary for HDV packaging and release.<sup>87</sup> Recently, several studies have attempted to reveal at a molecular level the role of HBsAg in HDV infection. While the large envelope protein has been shown to bind specifically to the cellular receptor NTCP<sup>9</sup> and, thus, be essential for HBV and HDV infectivity,<sup>72,88</sup> the presence of the small envelope protein appears to be sufficient for HDV packaging and release.<sup>87</sup>

The HDAGs are predominant in the nucleus of the infected hepatocyte<sup>89</sup> but can be post-translationally modified (e.g.

acetylation, isoprenylation, and phosphorylation).<sup>90</sup> Isoprenylation of the large HDAG was shown to lead to its translocation to the ER,<sup>91</sup> where it promoted HDV assembly by directly interacting with the small HBsAg (Table 1).<sup>92,93</sup> Changes in certain tryptophan-rich motifs in the carboxyl terminus of the small envelope protein of HBV<sup>79</sup> and within other distinct HBV envelope protein regions<sup>92</sup> were shown to affect such protein-protein interactions and negatively influence the assembly capacities of HDV. Hence, the emergence of HBV and/or HDV variants may also explain the fluctuating activities of HDV observed in the longitudinal study by Schaper *et al*.<sup>77</sup>

Huang *et al*. suggested that accumulation in the ER of all three HBV envelope proteins, especially of the large HBsAg, may increase the translocation of the large HDAG from the nucleus to the ER. This process, however, not only mediated HDV packaging but also caused ER stress.<sup>94</sup> Treatment of human hepatoma cells with two different ER stress inducers, brefeldin A and tunicamycin, was shown to promote the translocation of the large HDAG in the absence of HBsAg, at least in some cells (9%). During the same *in vitro* experiments, the HBsAg-induced translocation of the large HDAG was accompanied by an increase in nuclear nuclear factor kappa B (NFκB) activity, while inhibition of nuclear NFκB was shown to retain the large HDAG in the nucleus. In agreement with these observations, NFκB activation induced by tumor necrosis factor alpha (TNF-α) strongly promoted HDAG translocation in 50% of the cells, suggesting that ER stress and NFκB play an important role in the interaction of HBV and HDV during the stage of HDV assembly (Table 1).<sup>94</sup>

**Table 1. Interaction between HBV, HDV, and the infected target cell**

Interaction	Effect	Reference
HDV-HBV interaction		
L-HDAG ↔ S-HBsAg	HDV assembly	92, 93
S/L-HDAG ↔ HBV enhancers Enh1/2	HBV suppression	97
HDV-interaction with host factors		
L-HDAG ↔ DNA dependent RNA-polymerase II	HBV suppression	96
L-HDAG ↔ genes of SRE-dependent pathway	HBV suppression	95
S/L-HDAG ↔ MxA	Induction innate immune responses, HBV suppression	97
HDV ↔ ISGs, signaling genes, cytokines	Induction innate immune responses, HBV suppression	107
HBV-interaction with host factors		
HBsAg ↔ NFκB, TNF-α, ER-stress pathway	Translocation of HDV from nucleus to ER, HDV assembly	94
HBsAg ↔ TLR signaling	Suppression TLR signaling	110, 111
HBeAg ↔ TLR2	Impairment innate immune responses	109
HBV ↔ cellular DNA methyltransferases	Epigenetic modifications	115, 116
HBx protein ↔ innate signaling pathways	Impairment innate immune responses	117
HBV ↔ STAT translocation	Impairment IFN signaling	112, 113

### Interactions between HBV and HDV: role of the HDAG

Small and large HDAGs are not only involved in HDV replication and packaging, respectively, but they also appear to interact with HBsAg on an indirect molecular level, whereby they may be able to influence HBV replication.<sup>95-97</sup>

The large HDAG is known to be involved in HDV packaging by directly binding HBV surface proteins through its hydrophobic packaging signal<sup>98</sup> and its hydrophobic farnesylated C-terminal domain,<sup>99</sup> while the small HDAG binds to the HDV RNA and promotes HDV replication. An *in vitro* study by Wang *et al.* suggested that this HDAG-HDV RNA ribonucleoprotein complex also enhanced efficacy of HDV assembly, probably because the interaction between the large HDAG and HDV RNA was increased in the presence of the small HDAG (due to additional binding sites).<sup>43</sup> Indirect molecular interactions between HDAG and HBV were described by Modahl *et al.* in 2000, who suggested that the suppressive effect of HDV on HBV replication could be mediated by the large HDAG, which might inhibit not only the replication of HDV but also the transcription of the cccDNA by interacting with the host DNA-dependant RNA polymerase II.<sup>96</sup> Investigation of the interplay between HBV and HDV in a hepatoma cell line cotransfection model suggested that both the small and large HDAG were able to repress the two HBV enhancers, termed Enh1 and Enh2.<sup>97</sup> Of note, the same study also indicated that the large HDAG can activate the MxA promoter, thereby potentiating the effect of interferon- $\alpha$  on this cellular promoter. Moreover, the same study also suggested that HBV replication may be then inhibited by the antiviral activity of the interferon- $\alpha$  inducible MxA protein.<sup>97</sup> Goto *et al.* showed that the HBxAg and the large HDAG can synergistically activate the serum response element (SRE)-dependent pathway, which is involved in the expression of various genes that regulate cell growth, differentiation, and transformation. The synergistic induction of pathways involved in carcinogenesis<sup>95</sup> could in part explain the more severe course of HDV associated liver disease and why HBV/HDV coinfecting patients developing primary HCC are usually younger than HBV monoinfected individuals.<sup>100</sup> Also Wei and Ganem used an *in vitro* approach to show that the large, but not the small HDAG, has the capacity to activate heterologous gene expression by acting on a variety of promoters, including the pre-S, S, and C promoters of HBV.<sup>101</sup>

In summary, several possible interactions between HBV and HDV have been highlighted (Table 1). Many *in vitro* and *in vivo* studies, as well as clinical observations, have indicated that HDV is able to suppress the replication of HBV at certain time points of the coinfection. In this regard, the large HDAG seems to play a major role in mediating HBV suppression. Moreover, the fact that HBV surface antigens can mediate and increase HDV assembly<sup>92,93</sup> demonstrates the dependence of HDV on HBV as its helper virus and underscores the necessity of HDV to maintain HBV surface protein productivity. Although different interacting mechanisms have been suggested, further studies are needed to understand the impact of specific virus-virus interactions in the course of chronic infection, while a better understanding of the complex HBV/HDV interplay might even help identify new therapeutic strategies to cure chronic HBV and HDV infections.

### Interactions between HBV/HDV and human hepatocytes

Because there are only a few models available to study chronic infections with HBV and HDV in human hepatocytes, and biopsies of infected patients are rarely available, knowledge about interactions between HBV and HDV and the infected hepatocytes is scant.

While HBV is regarded as a virus that under most conditions is not directly cytopathic to infected hepatocytes, data from chimpanzees and specific clinical cases suggested direct cytopathic effects of HDV on hepatocytes,<sup>102-104</sup> particularly in the acute hepatitis setting.<sup>105</sup> In comparison to HBV, HDV generally shows a more severe clinical course. Experimental studies in chimpanzees,<sup>106</sup> humanized mice,<sup>107</sup> and recent patient observations<sup>108</sup> have indicated that HBV does not induce a strong activation of the innate immune system or interferon stimulated genes (ISGs) in the acute and chronic status of infection.

Moreover, different lines of evidence indicated that HBV was able to circumvent the induction of immune responses of the host by several mechanisms.<sup>2</sup> Both experimental studies<sup>109</sup> and observations in chronic HBV infected patients<sup>110,111</sup> indicated that the activation of toll-like receptor signaling molecules is impaired, while studies *in vitro*<sup>112</sup> and in human liver chimeric mice showed<sup>113</sup> that HBV can limit the interferon alpha mediated nuclear translocation of signal transducer and activator of transcription (STAT) (Table 1). *In vitro* studies also reported that HBV can interfere with STAT methylation<sup>114</sup> and activity of cellular DNA methyltransferases,<sup>115,116</sup> while HBV proteins, such as HBx protein, were shown to affect innate immunity pathways by downregulating mitochondrial antiviral signaling proteins (Table 1).<sup>117</sup>

In humanized mice, establishment of a stable HBV/HDV coinfection was shown to provoke a significant and sustained enhancement of the innate defense mechanisms in human hepatocytes compared to HBV infected animals.<sup>107</sup> Classic human ISGs and broadly acting effectors of the innate antiviral responses, such as Rig-I as well as STAT transcription factors, which are key signaling molecules that can be activated through direct viral actions,<sup>118</sup> were significantly higher in the setting of a chronic HDV infection.<sup>107</sup> Also, the expression of genes involved in antigen presentation and in recognition of infected cells by NK cells (e.g. hHLA-E, and hTAP1) was significantly increased compared to uninfected and HBV monoinfected mice.<sup>107</sup> This strong antiviral state caused by HDV observed in this study could also affect HBV replication in coinfecting livers (Table 1) and, hence, may in part explain the lower levels of HBV infection, which were frequently found in experimental approaches and in HBV/HDV coinfecting patients.<sup>62-66</sup>

### Interactions between HBV/HDV and the immune system of the host

HBV persistence is also associated with defective T cell responses characterized by suppression, dysfunction, and exhaustion of HBV-specific T cells, which appears to be provoked by dysregulation of costimulatory pathways, impairment of T cell receptor signaling, and enhanced T cell apoptosis.<sup>119</sup> However, HDV infected patients advance more rapidly in their disease than patients infected with HBV or HCV,<sup>34</sup> and clinical observations indicated that the liver damage associated with chronic HDV infection and the

severe course of infection seemed mainly to be immune-mediated. The small HDAg is thought to be responsible for the direct cytopathic effect on human hepatocytes, while the large HDAg seems to be noncytotoxic but promotes the persistence of HDV and may make hepatocytes susceptible to immune-mediated damage.<sup>39,102</sup> Although both innate and adaptive immune responses are believed to contribute to the pathogenesis of an HDV infection, detailed host immune responses in HDV infection are poorly investigated to date.<sup>39</sup> One very early study suggested that NK cells were activated upon interferon treatment in HDV infected individuals.<sup>120</sup> A more recent analysis in HDV infected patients revealed elevated levels of peripheral blood NK cells with a reduced functional capacity in their ability to respond to interferon- $\alpha$  treatment compared to healthy donors. A high frequency of NK cells before and during interferon treatment was positively associated with treatment outcome.<sup>121</sup> Moreover, *in vitro* experiments showed that the large HDAg might be able to activate STAT3 and NF- $\kappa$ B signaling<sup>97,122</sup> and that HDV interfered with interferon- $\alpha$  signaling by blocking the activation and translocation of STAT proteins, thereby contributing to the persistence of HDV and impairing therapy outcomes.<sup>123</sup> It was also suggested that this activation of STAT3 and NF- $\kappa$ B signaling by the large HDAg not only caused ER stress and necroinflammation but also increased the production of reactive oxygen species (ROS), possibly leading to the development of HCC.<sup>39</sup> In this regard, HDAg-induced STAT3 seemed to be able to activate DNA methyltransferases, which are known to silence tumor suppressor genes and lead to the development of HCC. Furthermore, HCC is often associated with an overexpression of clusterin. Interestingly, the large HDAg was shown to increase histone H3 acetylation of clusterin promoters and cell survival potential.<sup>39</sup>

Adaptive immune responses in HDV infections are, in general, weak.<sup>121</sup> In chronic HDV infected patients, responses of helper T cells are associated with a high frequency of secreting interleukin-10, which has immunomodulatory effects and inhibits interferon pathways.<sup>124</sup> In addition, perforin-positive cytotoxic CD4+ T cells, which are linked with killing infected cells, accumulated in chronically infected patients, and this might explain the more severe course of HDV associated liver diseases.<sup>125</sup> CD8+ T cell responses seemed to be weaker and were only detected in patients with past, but not active, HDV infections.<sup>126</sup> Impaired T cell responses observed in the setting of a chronic HBV/HDV coinfection occurred possibly due to the presence of HBV and its association with defective T cells responses. Since the activation of immune cells appeared to be limited in chronically HDV infected patients, it seems plausible that HDV exerts most of its effects in the liver itself, where the virus can mediate intracellular changes and can initiate inflammatory pathways.

## Conclusions

The fluctuating virus profiles observed in HBV/HDV coinfecting patients clearly show that the course of infection can be highly dynamic. Moreover, *in vitro* and *in vivo* experiments indicated that HDV is able to suppress HBV and even HCV replication.<sup>57,61–70</sup> As a consequence, HDV often appears as the predominant virus in coinfecting individuals.<sup>77</sup> In this regard, *in vitro* studies indicated that both the small and large HDAg proteins can be responsible for the reduction of HBV activity, since interactions of these proteins with host polymerases and HBV enhancers, as well as the induction of antiviral

ISGs and genes involved in cell growth and differentiation, have been described.<sup>95–97</sup> *In vivo* HBV/HDV coinfection of human hepatocytes was also shown to upregulate antiviral ISGs, signal molecules of innate immune response cascades, and cytokines more greatly than HBV alone.<sup>107</sup> Such proinflammatory status may explain the more severe course of disease in HDV infected patients. Since HBV acts as a helper virus by supplying its envelope proteins to HDV, productive HDV infections are strongly dependent on the expression of HBV envelope proteins. It is thus not surprising that, at least in certain phases of the infection, the levels of circulating HBsAg correlate with HDV RNA levels.<sup>76,77</sup> *In vitro* studies also proposed that HBsAg promotes nuclear export of the large HDAg and HDV assembly by inducing ER stress, nuclear NF $\kappa$ B translocation, and TNF- $\alpha$  production; thus pointing out the existence of complex cross-talk among HBV, HDV, and the infected cell.<sup>72,94</sup> Cell-mediated prenylation of the large HDAg is also important for the efficient translocation and assembly of HDV.<sup>91–93</sup> Since this step appears to be crucial in the HDV life cycle, prenylation inhibitors, such as lonafarnib, are currently being tested in clinical studies. The use of HBsAg inhibitors may also represent an interesting approach for targeting HDV assembly, since a strong decrease of HBsAg levels is expected to affect the amounts of circulating delta virions.

The availability of innovative experimental systems and techniques, which allow for more sensitive molecular analyses of these viruses and of the host, has begun to provide new insight into the complex network of virus-host interactions that are established in the course of viral hepatitis infection and therapy. Nevertheless, because of the limitations encountered by using any experimental model, studies based on human blood and liver biopsy samples remain indispensable to gain insights about the pathobiology of HBV and HBV/HDV infection and for the development of effective HBV/HDV therapies that will permit achievement of a functional HBV cure and HDV eradication.

## Conflict of interest

None

## Author contributions

Writing the manuscript and designing the figure and table (KG, MD).

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