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Commentary Open Access

Commentary to: Relative Abundance of Integrant-Derived Viral RNAs in Infected Tissues Harvested from Chronic Hepatitis B Virus Carriers by Freitas N, et al. 2018

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Commentary

During infection with Hepatitis B Virus (HBV), viral DNA is able to integrate into the chromosomal DNA of infected hepatocytes. The main substrate for the integration is double-stranded linear DNA genome of HBV (DSL), which represents approximately 5-20% of the final products of the reverse transcription catalyzed by the viral polymerase, while the major portion of the final products of HBV reverse transcription is represented by relaxed circular DNA genomes (rcDNA) (Figure 1). The HBV DNA integration is a by-product of viral replication and it is random. Over time, a significant number of infected hepatocytes can acquire HBV DNA integrants [1-3]. Since integrated HBV DNA cannot serve as the source of the progeny virus, it has been commonly assumed that the HBV life cycle is independent of HBV DNA integrants. Using liver and matching hepatocellular carcinoma (HCC) tissues harvested from individuals that were chronically infected with HBV and that were not treated with anti-HBV drugs, our recent study demonstrated that RNA transcripts arising from integrated HBV DNA (i.e., HBV integrant-derived RNAs or id-RNAs) were abundantly or even predominantly present, when compared to viral RNAs transcribed from the episomal, covalently-closed circular viral DNA (cccDNA) (i.e., replication-derived or rd-RNAs) [4]. The observed relative abundance of id-RNAs suggested that these RNA molecules could serve as a considerable source of the HBV envelope proteins (or surface antigen, HBsAg) that is independent of the HBV genome replication. Furthermore, our study suggested that id-RNA-derived HBsAg (i.e., id-HBsAg), when abundant as compared to rd-RNA-derived HBsAg (i.e., rd-HBsAg), can regulate the assembly and infectivity of HBV virions, and thus can influence the ability of HBV to support virus spread and super-infection (Figure 1). The data further suggested that id-RNAs/id-HBsAg therefore can possibly influence the maintenance of the chronic state of HBV infection. In addition, the study proposed to revise the current model of the HBV life cycle by incorporating the potential contributions of id-RNAs and id-HBsAg as described above [4]. Moreover, it became apparent that the inability of current anti-HBV drugs to achieve loss of serum HBsAg in the vast majority of treated patients can be likely explained by significant amounts of id-HBsAg that are translated from id-RNAs independently of HBV replication [4]. Consequently, it is reasonable to suggest that the abundant presence of id-RNAs/id-HBsAg during chronic HBV infection may also have important implications for HBV-related liver pathogenesis (including carcinogenesis), co- or super-infection with Hepatitis Delta Virus (HDV) [4], and innate and adaptive immunity (Figure 1). The above findings also indicate that the development of new therapeutic interventions directly targeting id-RNAs and/or id-HBsAg may soon become an important task for the management of chronic HBV infection.

HBV Pathogenesis and Carcinogenesis

High levels of circulating and intracellular HBsAg can mediate serious liver pathogenesis in HBV-infected patients, including the development of cirrhosis, hepatic decompensation, and HCC [2,5]. Contrary, the risk of developing HCC is significantly reduced in individuals who spontaneously cleared HBsAg from the circulation (in addition to HBV DNA) or who achieved undetectable serum viremia and antigenemia levels during treatment [6,7]. In regard to these reports, although not demonstrated so far, abundant id-HBsAg, in addition to rd-HBsAg, may substantially account for HBV-associated liver disease progression. Since decades it is known that integrated HBV DNA is present in non-malignant liver tissues as well as in HCC tissues of the same patients [1]. Compared to non-malignant liver tissue from the same patients, a recent report found that HBsAg is differentially expressed in HCC tissues [8]. Although this study did not address the source of the viral protein (i.e., rd- versus id-HBsAg), the expression of the large form of HBsAg (LHBs or the large envelope protein) was unchanged, whereas expression of the small form of the protein (SHBs) was reduced or absent in HCC. Consistent with studies in mouse models of HBV [9,10], these results indicate that continuously produced wild-type (or mutated) LHBs in the absence of SHBs may accumulate in the endoplasmic reticulum of hepatocytes leading to cellular stress and cytopathic effects thereby promoting HCC development. Mutated LHBs has been indicated in the development of mouse ground glass hepatocytes, which cluster and form nodules and are seen as preneoplastic lesions [9]. Related to this finding, the clonal outgrowth of hepatocytes with limited or undetectable HBV replication, which are not targeted by the immune system, has been implicated in HBV-associated carcinogenesis [3,11,12]. Furthermore, since it was shown in cell culture that HBsAg can suppress intracellular HBV genome replication [13], it remains an interesting possibility that at least some hepatocytes involved in this clonal outgrowth may have considerably reduced levels of HBV replication, because of the production of id-HBsAg. Thus, an immune-driven liver repopulation with initially non-malignant hepatocytes could facilitate HCC onset, when these cells produce id-HBsAg for suppression of HBV replication, and id-HBsAg accumulates overtime within these cells. In addition, it can be speculated that continued production of HBV virions in such hepatocytes, which bear id-HBsAg rather than rd-HBsAg as a major component of their outer envelope, albeit at a very low level, may represent one of the strategies adopted by HBV to maintain chronic infection in the face of immune surveillance,

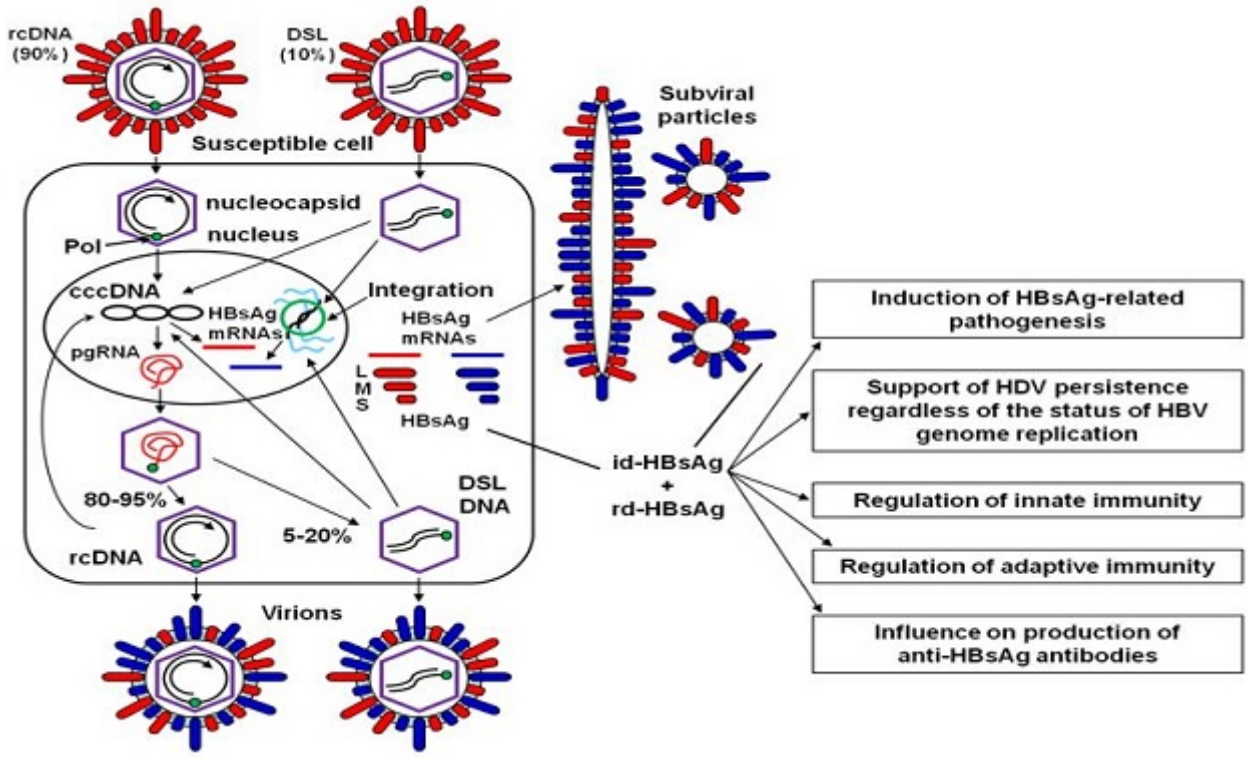

Figure 1: Integrant-derived envelope proteins of HBV (id-HBsAg) and their role in the life cycles of HBV and HDV, HBsAg-related pathogenesis, and interactions with the host immune responses. The presented on the left model of the life cycle of HBV that incorporates production of id-HBsAg and its potential roles and functions is based on the one that was published by Freitas N, et al [4]. About 90% of HBV virions bear relaxed circular DNA genome (rcDNA) inside the nucleocapsids. The other ~10% contain double-stranded linear DNA genome (DSL). The HBV entry is a receptor-mediated endocytosis. During the uncoating inside the cell, virion loses the envelope that consists of three viral envelope proteins, large (L), middle (M) and small (S) (shown as bars), and some lipid. Next, DNA synthesis resumes inside the nucleocapsid, and plus DNA strand becomes completed. The polymerase (i.e., reverse transcriptase) of HBV is indicated as a small green circle. After the entry into the nucleus, the nucleocapsid gets disassembled. The rcDNA loses the polymerase, and subsequently gets converted into covalently closed circular DNA (cccDNA) with the help of the host enzymes. The newly-formed cccDNA is the template for synthesis of all RNAs of HBV. One of the RNAs produced by HBV genome replication is pre-genomic RNA (pgRNA). The pgRNA in the complex with viral polymerase gets incorporated into the nucleocapsid made out of HBV core protein molecules. Next, the reverse transcription begins inside the nucleocapsid. The major final product of the reverse transcription (~80-95% of cases) is rcDNA. However, in 5-20% of cases, the final product of the reverse transcription is DSL. Encapsidated rcDNA or DSL can undergo the envelopment with HBsAg and then be released from infected cell as HBV virions. Alternatively, they can be delivered to the nucleus. Inside the nucleus, rcDNA (as mentioned above) is converted into cccDNA. This is how the cccDNA pool gets replenished. Furthermore, DSL is the main substrate for HBV DNA integration. The integration occurs randomly into the host chromosomal DNA and is facilitated by the host DNA repair enzymes [1-4]. Integrated DSL can function as the template for the transcription of HBV integrant-derived RNAs (id-RNAs) encoding HBsAg (independently of HBV genome replication) [4]. The mRNAs for HBsAg are shown as two horizontal lines. The red line represents all HBV replication-derived (i.e., cccDNA-derived) mRNAs (rd-RNAs) coding for HBsAg. The blue horizontal line represents all id-RNAs coding for HBsAg. The L, M and S indicate the large, middle and small envelope proteins of HBV, respectively. Shown is the situation, when in infected cell, mRNAs for HBsAg are generated by virus replication and are also produced from integrated HBV DNA. In this case, the envelope proteins are produced from rd-RNAs and from id-RNAs. When id-RNAs are relatively abundant, the newly formed virions of HBV will bear id-RNA-derived HBsAg (id-HBsAg, shown as blue bars) as a major component of their envelopes. Therefore, the properties of these HBV virions will be mainly defined by the functional properties of id-HBsAg, and not by the properties of rd-RNA-derived HBsAg (rd-HBsAg, shown as red bars). Accordingly, id-HBsAg (when abundant) can determine the capability of HBV virions to support virus spread and super-infection that could influence the HBV ability to maintain chronic infection [4]. Also shown are subviral particles that are composed of id-HBsAg and rd-HBsAg, and are released in huge excess as compared to the numbers of HBV virions. Subviral particles come in the forms of small spheres or filaments of various length [2]. Also indicated is the intracellular and secreted HBsAg (of both origins (i.e., id-HBsAg and rd-HBsAg)). On the right part of the figure are important processes that can be influenced by abundant intracellular and/or secreted id-HBsAg (i.e., HBsAg-associated liver pathogenesis, persistence of human Hepatitis Delta Virus (HDV) that uses HBsAg to form its own virions, innate and adaptive immune responses, and production of anti-HBsAg antibodies).

if the immune system does not efficiently recognize id-HBsAg. Interestingly, one study, which analyzed serum samples from individuals chronically infected with HBV, suggested that circulating antibodies against HBsAg (HBsAb) did not bind HBsAg, since the HBsAg subtypes present in serum did not display the determinants against which these antibodies were developed, and that this could have prevented the formation of immune complexes [14].

HDV Infection

Co- and super-infection with HDV contributes substantially to HBV pathogenesis and carcinogenesis, since this virus mediates liver injury in addition to HBV, induces faster and more frequent development of cirrhosis, and thus increases the overall risk of HCC in HBV/HDV-infected individuals [15-17]. As a subviral agent of HBV, HDV needs only HBsAg from HBV, but otherwise its life cycle is independent of HBV [17]. Furthermore, persistence of HDV could be independent of HBV replication as previously hypothesized by us [18]. This hypothesis is now further supported by the data from our new study indicating that HBsAg needed for the envelopment of HDV virions may be translated in vivo from id-RNAs (Figure 1) regardless of HBV genome replication [4]. In vitro, we have previously shown that id-HBsAg in the absence of rd-HBsAg supports the production of infectious HDV virions [18]. Use of id-HBsAg rather than rd-HBsAg by HDV could also explain the failure of current anti-HBV drugs to efficiently interfere with HDV infection [19]. Since no drug is available that directly blocks HDV infection [16,19], this underlines the importance of exploring id-RNAs and/or id-HBsAg as targets for the therapeutic intervention of HDV, as well as of HBV, as discussed below.

Innate and Adaptive Immunity

Several reports suggest that circulating HBsAg may directly regulate innate immunity. HBsAg can suppress plasmacytoid dendritic cell function and affects the transcription of interferon-alpha (IFN-α) mediated by activation of the toll-like receptor 9 [20]. Internalization of HBsAg by myeloid dendritic cells is associated with a diminished upregulation of costimulatory molecules and reduced T cell stimulatory capacity [21]. HBsAg has been also shown to suppress the activity of monocytes and to affect cytokine production [22]. Furthermore, the number of natural killer cells declines and cytotoxic cell function becomes attenuated with the expression of HBsAg in the liver of HBV transgenic mice [23]. Circulating HBsAg also affects adaptive immunity since persistent exposure of CD4+ and CD8+ T cells to this protein leads to a stepwise and progressive loss of their effector functions resulting in the overall exhaustion and even deletion of these immune cells [24-26]. Functional impairment of host immunity by HBsAg then in turn renders HBV-infected individuals unable to mount an effective antiviral response that is a prerequisite for HBsAg clearance. The contribution of id-HBsAg in the dysfunction of the above immune cells has not been investigated so far. However, it is known that patients with chronic HBV infection display diminished T cell responses to HBsAg [27], and that reductions in the levels of rd-HBsAg by treatment is associated with recovery of this T cell function [28].

As mentioned above, HBV-infected patients with advanced liver disease and HCC often present with mutations of HBsAg. These commonly found deletions correlate with epitopes affecting CD8+ T cell response and B cell neutralization [29], and thus may help the virus to escape the antiviral immune response. Although not tested as of yet, it is conceivable that id-HBsAg in mutated form, in addition to mutated rd-HBsAg, may contribute to the degree of immune evasion. In this regard, the involvement of humoral response in the control of chronic HBV infection is not very clear. Neutralizing HBsAb are induced in individuals following recovery from acute HBV infection or after immunization with standard vaccines based on recombinant HBsAg, and can inhibit viral attachment and entry [30]. As discussed below, seroconversion to HBsAb is considered a desired endpoint of anti-HBV treatment in individuals with chronic HBV infection. A direct suppression of B cell functions by HBsAg is not known; however, HBV-infected individuals can present with “hyperactive” B cells that show upregulated activation markers and impaired functions [31], and with memory B cells that display reduced proliferative capacity [32]. Thus, (memory) B cell dysfunction may contribute to the immune evasion by HBV.

This overall suggests that high levels of HBsAg can result in the impairment of the host immune response at several distinct levels (Figure 1). Rendering the host unable to mount an effective antiviral response required for clearance of rd- and id-HBsAg may represent a strategy adopted by HBV to maintain the status of chronic infection in the presence of immune surveillance. Reductions in the level of rd-HBsAg have been implicated in the reconstitution of antiviral immune functions in HBV-infected individuals, which has implications for novel interventions against chronic HBV infection.

Development of Therapeutic Interventions for id-RNAs and id-HBsAg

Current FDA-approved treatment options for chronic HBV infection include five nucleos(t)ide analogs (NUCs) and pegylated IFN-α. NUCs are inhibitors of the HBV reverse transcriptase function. They efficiently affect viral genome replication in hepatocytes and can reduce circulating serum HBV DNA to undetectable levels after prolonged treatment. However, the majority of treated patients do not achieve serum HBsAg loss and subsequent seroconversion to HBsAb [33]. Sustained undetectability of viremia and antigenemia (with or without seroconversion) after completion of treatment is a desired endpoint of HBV therapy, since it indicates that the immune system has gained control over the virus leading to a “functional cure” of HBV infection [33]. The reason for the rare HBsAg loss after treatment completion is unclear, but may relate to the narrow mechanism of action of NUCs (i.e., primarily suppression of HBV DNA synthesis) [34]. Another possibility, as discussed above, is that significant HBsAg amounts in patients may be derived from id-RNAs, in addition rd-HBsAg (Figure 1). This assumption is supported by a recent study in HBV-infected patients that demonstrated only modest changes in HBsAg serum levels despite undetectable cccDNA in liver following long-term NUC treatment [35]. In line with this finding, the levels of intracellular and circulating id-HBsAg are not expected to markedly change during current antiviral treatment, since NUCs do not directly target id-RNAs. IFN-α also does not directly affect id-RNAs or id-HBsAg, but due to its pleiotropic effects on viral and host factors, this cytokine facilitates immune-mediated killing of HBV-infected hepatocytes. Since IFN-mediated removal of HBV replicating hepatocytes also includes those cells with integrated HBV DNA, id-HBsAg levels may decline over time, and faster than commonly observed with NUCs. This underlines the need of new therapeutic interventions that directly target id-RNAs for reducing HBsAg, which is independent of rd-RNAs, for treatment of HBV, as well as HDV. Significantly reduced id-HBsAg levels would then contribute to the overall loss of circulating HBsAg thereby allowing restoration of innate

and adaptive immunity. This could be achieved by treatment with monoclonal antibodies that target specifically id-HBsAg, in case that B cell epitopes are different to those present on rd-HBsAg. Otherwise, antibodies could be used to neutralize HBsAg of both sources from the blood compartment [36]. Expression or secretion of id-HBsAg from hepatocytes may be further inhibited with compounds such as antisense oligonucleotides, small interfering (si)RNA, nucleic acid polymers, and/or synthetic small molecules, which are currently investigated for efficacy against rd-RNA and rd-HBsAg [37-41].

Outcome Expectations for New Therapies Directly Targeting id-RNA and id-HBsAg

As outlined above, a profound suppression of both sources of HBsAg (i.e., rd-RNAs and id-RNAs) will help achieving the highly desirable outcome of the current guidelines on the management of chronic HBV infection - loss of serum HBsAg [42]. Since loss of HBsAg is considered crucial in the overall restoration of immune functions and in decreasing the risk of associated liver disease progression and HCC, inhibitors of id-RNA/id-HBsAg will most likely need to be combined with conventional NUC treatment or IFN-α. Such combinations may induce not only "functional cure", but may be also able to extend the desired treatment outcome into a "complete cure" [42]. In this scenario, rd-and id-HBsAg become undetectable, subsequent seroconversion to HBsAb is induced, and intrahepatic cccDNA are eradicated following finite treatment duration, all of which would substantially lower the risk of viral reactivation and disease progression.

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