Chapter 13
Occult HBV Infection

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Introduction

Hepatitis B virus (HBV) occult infection is defined as the presence and long-lasting persistence of viral DNA in the liver (with detectable or undetectable HBV DNA in the serum) of individuals testing negative for the HBV surface antigen (HBsAg) [1]. Apart from some cases in which the lack of HBsAg detection is attributable to the HBV genetic heterogeneity, i.e., infection with S-escape mutants producing a modified HBsAg that is not recognized by the commercially available diagnostic assays [2, 3], in most cases the occult HBV infection (OBI) is due to replication-competent viruses with degrees and relevance of genetic heterogeneity comparable with those of the HBV isolates from individuals with HBsAg positive (namely "overt") infection [4]. In OBI cases, however, the viruses are subjected by the host's defense mechanisms to a potent suppression of the replication activity and gene expression, leading to the lack of both HBsAg synthesis and production/secretion of virions and thus to the absence (or presence in minute traces) of HBV DNA in the serum [5, 6].

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The molecular basis of the occult infection is strictly related to the peculiar life cycle of the HBV, and in particular to the high stability and long-term persistence of viral cccDNA molecules in the nuclei of the hepatocytes that—together with the long half-life of the liver cells—imply that, once the HBV infection has occurred, it may possibly continue for life [7]. Indeed, according to the European guidelines on HBV management, OBI is recognized as one of the five phases of the natural history of chronic hepatitis B [8]. In this context, it is important to stress that HBV DNA may be found integrated into the host's genome in each of these five phases regardless of the HBsAg positive/negative status. Viral DNA integrants have no role in the HBV life cycle, and their possible presence in HBsAg-negative subjects does not per se have to be identified as an occult infection since OBI is essentially related to the intrahepatic persistence of entire, episomal, replication-competent HBV genomes.

Suspected for several decades of existing (reviewed in ref. [5]), the occult phase of the HBV infection was better identified in the late 1990s when some important clinical-virological studies (based both on the analysis of well-selected and characterized human liver samples and on the application of highly sensitive molecular biology techniques) made it possible to start revealing its potential implication in various clinical contexts, to show its worldwide diffusion, and to disclose its virological aspects [9]. Indeed, in recent years there has been a continuous increase of the number of studies in this field published by journals covering different areas of biomedical interest (reviewed in ref. [5]), thus making OBI one of the most challenging and fascinating issues of the research into viral hepatitis.

Mechanisms Leading to Occult HBV Infection Development

Major advances have been made in the last few years in understanding the molecular mechanisms potentially involved in the induction and maintenance of the HBV infection in an occult status. Although viral factors may be implicated in some cases, host factors (immune response and epigenetics) likely play a preeminent role (Fig. 13.1), and there is evidence that coinfection with other viral and nonviral agents might also be involved in some circumstances [5, 6, 10].

Viral Factors

The lack of detectable HBsAg in spite of the presence of episomal, free HBV genomes at intrahepatic level is attributable in some cases to the HBV genetic variability. In fact, a fairly large number of studies have linked OBI occurrence to specific HBV variants. Indeed, it has been reported that OBI individuals are infected with HBV variants showing (a) mutations clustering in major hydrophilic region (MHR) of the small (S) protein, (b) mutations in the pre-S1/S2 genomic region, (c) specific structural alterations in virus regulatory elements, (d) mutations affecting posttranslational production of virus envelope proteins, and (e) mutations selected under antiviral treatment with nucleos(t)ide analogs (NUCs) that may cause amino acid changes both in

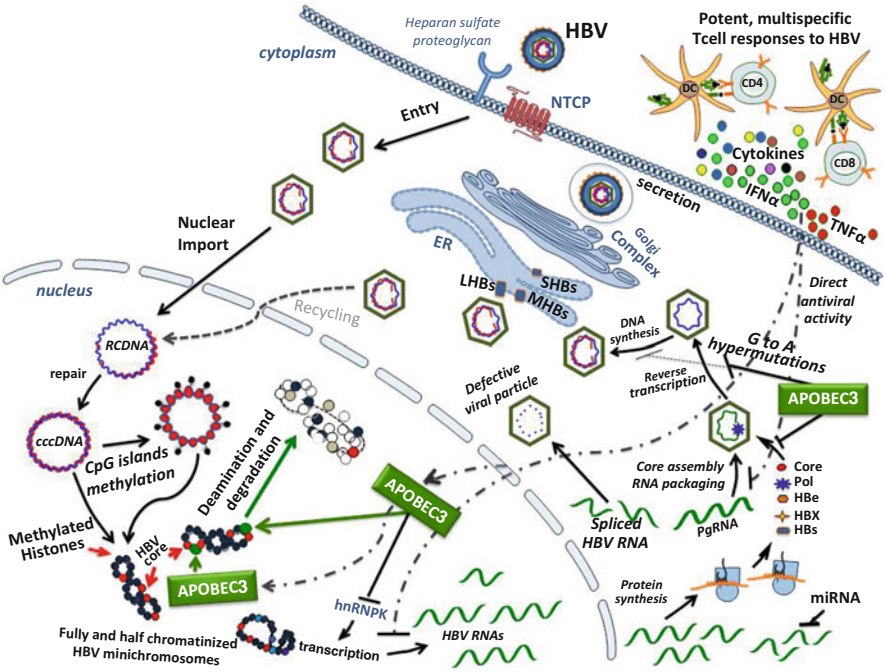

Fig. 13.1 Schematic representation of the main mechanisms leading in the control of HBV activities and potentially involved in OBI occurrence. In particular, mechanisms related to host's adaptive (functionally efficient memory HBV-specific T cell response) and innate immune response (cytokines like IFN-α and TNF-α) as well as genetic (APOBEC3 hyperediting resulting in HBV genomic hypermutation, apurinic/apyrimidinic site formation, and cccDNA degradation), epigenetic (methylation of HBV CpG-islands and cccDNA-bound histones tails, full nucleosomal packaging of HBV minichromosome) and co-/posttranscriptional (cellular miRNAs- and/or APOBEC3s [editing-independent]-induced inhibition of HBV replication, and HBV RNA splicing) mechanisms are summarized. *NTCP* sodium taurocholate cotransporting polypeptide, *DC* dendritic cell, *CD4* CD4+ T cell, *CD8* CD8+ T cell, *RC DNA* relaxed circular DNA, *cccDNA* covalently closed circular DNA, *pgRNA* pregenomic RNA, *LHBs* large hepatitis surface protein, *MHBs* middle hepatitis surface protein, *SHBs* small hepatitis surface protein, *APOBEC3* apo B mRNA editing enzyme catalytic polypeptide, *hnRNPK* heterogeneous nuclear ribonucleoprotein K, *miRNAs* microRNAs

viral polymerase and S protein [5, 10–12]. A high frequency of mutations has been found particularly within the MHR of HBsAg in HBV strains isolated from OBI individuals [4, 11, 13–24]. These mutations have been functionally associated with S protein structural changes that may lead to an impaired detection by commercially available HBsAg assays. In addition, there is evidence that occult HBV of specific genotypes may show not only the mutations in the MHR, but also a very high frequency of mutations in the T-cell epitopes, thus further supporting the hypothesis that the selection of these HBV variants may represent a mechanism of immune escape, as also suggested by the inability of anti-HBs antibodies from individual patients to recognize their own circulating viruses [11, 19–21, 25, 26]. Some recent studies have strengthened these data by applying ultra-deep pyro-sequencing. Indeed, a higher

degree of genetic variability was found in the S gene of occult HBV compared to viruses from HBsAg-positive patients, and it has been postulated that the complex HBV quasi-species with mutations in HBsAg immune-active regions may help HBV to escape both from neutralizing and diagnostic antibodies [24, 27]. However, this evidence has been challenged by a very recent study showing that the genetic heterogeneity of reactivated HBV is significantly lower in patients with reactivation from OBI carrier status than that from HBsAg-positive carriers, suggesting that OBI individuals are infected with HBV populations of low genomic heterogeneity in their liver [28]. The very low or absent viral load characterizing OBI carriers has suggested that viral genomic mutations could also negatively impact any step of HBV life cycle [21, 23, 29, 30]. Indeed, there are data showing that occult HBV with specific amino acid substitutions in the MHR displays an impaired virion and/or S protein secretion when transfected in hepatoma cells or hydrodynamically injected in mice [21, 23, 30]. In this context, it is worth mentioning that also mutations at the level of the pre-S2/S splice donor site have been detected in occult HBV strains. Pre-S2/S splicing occurs during HBV replication, and mutations that interfere with pre-S2/S mRNA splicing may cause a marked reduction of functional unspliced pre-S2/S transcripts and of HBsAg synthesis, thus leading to OBI development. There is evidence that RNA secondary structure at the 5′ splice site can regulate the splicing efficiency of transcripts and modulate the binding of RNA-splicing factors as well as the recognition of splice site consensus elements [31]. Thus, it has been postulated that mutations at the pre-S/S 5′ splice donor site may affect the interaction of RNA with components of the spliceosome, hence impairing posttranscriptional RNA processing and/or nuclear export via the posttranscriptional regulatory element [25, 32, 33]. Pre-S mutations have also been associated with OBI occurrence. In particular, it has been shown that deletions in the pre-S1/S2 genomic region correlate with an impaired expression of envelope proteins, and that some of these deletions may contribute to persistence of the virus in the occult state by implying the elimination of HLA-restricted B-cell and T-cell epitopes [34–36]. The association of mutations and deletions in the pre-S gene with a lack of secreted HBsAg and low levels of HBeAg and HBV DNA was demonstrated using functional analysis by transfection into hepatocyte cell lines [36].

Despite all these lines of evidence, however, it is proved that the great majority of OBI individuals are not infected with specific HBV mutants. Moreover, important data have demonstrated that pre-S/S variants can frequently be found also in patients with overt HBV infection, including subjects with high viral loads [4, 12, 22, 35, 37]. Furthermore, strong evidence from different studies indicates that "occult" HBV genomes are usually replication-competent and that their genetic heterogeneity is comparable with those from HBsAg-positive individuals [4, 28, 37]. In vitro functional analysis showed that occult viral isolates "re-acquire" normal replication, transcription, and protein synthesis abilities once taken out from the host's liver microenvironment. These viruses appear to normally replicate when transfected in hepatoma cells and to be competent in HBsAg production [4]. Therefore, according to these findings genomic variability does not usually appear to play a fundamental role in inducing the OBI status, which rather seems to be dependent on a strong suppression of the virus replication and transcriptional capabilities in the majority of the cases.

Host Factors

Immunological Factors

Many clinical studies have provided strong evidence indicating that all the conditions inducing immunosuppression expose patients to risk of OBI reactivation with the reappearance of the typical serological profile of the overt, active HBV infection [5, 38–40]. Though indirect, this is strong evidence of the role played by the host's immune surveillance in OBI induction. The importance of the immune system in OBI occurrence has also been demonstrated by the findings showing that HBV DNA along with a functional memory HBV-specific T cell response can be readily detectable several years after recovery from an acute hepatitis B event [41, 42]. Thus, it is plausible to hypothesize that during the occult phase of the infection, HBV is still able to synthesize very small amounts of antigens that, however, are sufficient to maintain an HBV-specific T cell response. This assumption is confirmed by the findings showing that, apart from HBV covalently closed circular DNA (cccDNA) molecules [43–46], all viral HBV transcripts (including the pregenomic RNA, pgRNA) can also be detected and quantified in the liver of OBI individuals [44, 46]. Importantly, some recent studies have shown that OBI individuals can display a potent HBV-specific T cell response [22, 47]. In particular, it has been demonstrated that OBI patients with or without antibodies to HBV core antigen (anti-HBc) display different profiles of HBV-specific T cell responses. Indeed, although in anti-HBc negative (namely, seronegative) OBI patients circulating HBV-specific T cells can be detected at frequencies comparable with that found in anti-HBc positive (namely, seropositive) OBI subjects, in vitro expansion and IFN-γ production by HBV-specific T cells from seronegative cases are much weaker than those from OBI seropositive individuals [47]. On the basis of the data obtained in the woodchuck animal model infected with the corresponding hepadnavirus (woodchuck hepatitis virus, WHV), it has been hypothesized that these distinct behaviors of cell-mediated immune responses in seropositive and seronegative OBIs might reflect different modalities of HBV transmission. Indeed, exposure to low WHV doses (less than 10^3 virions) may lead to a persistent infection without appearance of viral serum markers. Interestingly, this so-called woodchuck "primary" occult infection does not confer protective immunity, indicating that only infection with a higher dose of inoculum can elicit an efficient memory T cell response [48]. Potent, HBV-specific T cell responses were also observed in blood donors with seropositive OBI [22]. Of interest, it was observed that HBV-specific T-cell responses could be quantitatively stronger in OBI than in inactive carriers, and similar or even higher than those in subjects with previously resolved hepatitis B [22].

Many relevant data have suggested that the innate immune response also may play a role in the control of HBV activities. Experiments in transgenic mice and chimpanzees have shown that inflammatory cytokines, such as type I interferons (IFN-I) and tumor necrosis factor-α (TNF-α), can efficiently suppress viral replication through noncytolytic immune response [49]. In accordance, it has been recently demonstrated that liver cells can mount an effective innate immune response to HBV infection with

the expression of IFN-stimulated genes, which in turn limit HBV replication via inhibition of cccDNA transcription and encapsidation of pgRNA [50]. Moreover, it has been shown that activation of the retinoic acid-inducible gene 1 (RIG-I) like receptors in infected hepatocytes induces the production of IFNs and different proinflammatory cytokines, and also activates intracellular antiviral pathways to disrupt HBV replication by targeting multiple steps of the viral life cycle [51].

Interestingly, recent studies have proved that the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 (APOBEC3) cytidine deaminases represent a major strategy of innate immunity to retroviruses as well as to the pararetrovirus HBV [52]. It has been shown that the expression of APOBEC3G in HBV-replicating cells results in more than a 50-fold decrease in HBV DNA release in the cell culture medium [53]. Both deamination-dependent and deamination-independent mechanisms have been implicated in APOBECs-induced inhibition of HBV replication [52]. Very recently it has been shown that IFN-alpha can up-regulate APOBEC3A in HBV-infected cells and that HBV core protein mediates the interaction of APOBEC3A with HBV cccDNA, resulting in cytidine deamination, apurinic/apyrimidinic site formation, and finally in cccDNA degradation [54]. Interestingly, APOBEC hyperedited sequences have also been detected in OBI individuals [35, 55]. Altogether, these findings indicate that the innate immune response may have a leading part in the control of HBV activities in OBI, and particularly in seronegative OBI patients in whom poor in vitro T cell expansion has been observed.

Epigenetic Factors

Recently, studies on the role of viral chromatin organization have revealed the importance of dynamic viral-host chromatin interactions in modulating the control of essential viral processes including gene expression and replication [56]. Many different chromatin-organizing factors have been associated with the epigenetic configuration of the viral chromosome. For DNA viruses like Epstein–Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) known to establish latent infection, the contribution of chromatin remodeling to the latent state has been investigated in depth. During latency, both EBV and KSHV genomes are maintained as minichromosome molecules that adopt a chromatin conformation similar to that of the host cell chromosome, and many data indicate that both viruses make use of chromatin binding factors and histone tail epigenetic modifications as mechanisms to maintain unchanged gene programs during latent infection [56–58]. Many recent studies have shown that epigenetic mechanisms play a relevant role also in controlling HBV transcription/replication [59, 60].

HBV cccDNA molecules are harbored in the nucleus of infected hepatocytes as stable minichromosomes displaying the typical "beads-on-a-string" structure at electron microscopy, and showing the DNA packed into the full or half complement of nucleosomes, which can reflect dynamic changes related to transcriptional activity [61–63]. HBV cccDNA minichromosomes associate with both histone and non-histone proteins [59]. Indeed, H1, H2A, H2B, H3, and H4 histones as well as the

viral core protein have been shown to be a structural component of the HBV minichromosome [61]. Data from transfected hepatoma cells and liver tissues have shown that HBV replication is regulated by the acetylation status of viral cccDNA-bound H3 and H4 histones, and that recruitment of histone deacetylase 1 (HDAC1) onto the cccDNA correlates with low HBV replication [64]. In addition, treatment with inhibitors of class I or class III HDACs induces a significant increase of the acetylation status of cccDNA-bound histones and HBV replication in HBV-replicating cells [64]. Interestingly, there is evidence demonstrating that IFNα is able to inhibit cccDNA-driven transcription of viral RNAs, both in HBV-replicating cells and in HBV-infected humanized uPA/SCID mice [65, 66]. In particular, it has been found that cccDNA-bound histones become hypoacetylated, and components of the transcriptional repressor complex PRC2 are actively recruited on the cccDNA after IFNα treatment [66]. Therefore, IFN-α appears to be capable of inducing a condition of "active epigenetic control" of HBV cccDNA minichromosome activity, which may have a part in the persistent (although reversible) "off therapy" inhibition of HBV replication. Of note, it has also been shown that the HBX regulatory protein produced in hepatoma cells replicating HBV is recruited onto the cccDNA minichromosome, and that HBx-defective HBV mutants are impaired in their replication [67, 68]. There is evidence that, in addition to chromatin dynamics, CpG site-specific DNA methylation levels in the HBV genome may also contribute in modulating viral gene expression and replication [5, 10, 59, 60]. Interestingly, DNA methylation analysis of a certain number of OBI cases revealed that specific CpG sites in the HBV genome are frequently hypermethylated [35]. However, more recent results have argued that in normal hepatocytes—unlike in hepatocellular carcinoma (HCC) cells—DNA methylation could be a major epigenetic mechanism responsible for chronic silencing of HBV gene expression [69]. Therefore, the exact impact of the observed CpG islands methylation on the function of HBV genome and occult infection remains to be established.

The contribution of cellular and viral micro-RNAs in regulating viral replication and chromatin is also under intense investigation. To examine cellular micro-RNAs affecting HBV replication, Zhang et al. applied a loss-of-function approach by transfecting antagomirs targeting many different human micro-RNAs in hepatoma cells [70]. Both miR-199a-3p and miR-210 have been found to suppress HBsAg expression. In addition, another study showed that also miR-125a-5p may interfere with HBsAg expression and release in the cell culture medium [71]. Recently, many cancer-related micro-RNAs, including miR-15a/miR-16-1, the miR-17-92 cluster, and miR-224, have been shown to target HBV mRNAs, thus inhibiting HBV replication [72–74]. Besides directly targeting HBV, some cellular micro-RNAs have been shown to inhibit HBV replication by indirectly regulating different cellular transcription factors. In particular, miR-141 has been shown to significantly suppress HBV expression and replication in HepG2 cells by targeting the peroxisome proliferator-activated receptor alpha [75], and miR-155 may impair HBV replication in hepatoma cells through targeting the suppressor of cytokine signaling proteins (SOCS1), and promoting the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway [76].

Coinfection

Several studies have shown that HBV replication is frequently impaired in individuals coinfected with other infectious agents. In particular, it has been shown that hepatitis C virus (HCV) infection can strongly suppress HBV replication, and this has led to hypothesize that the inhibitory activity exerted by HCV on HBV might ultimately result in OBI occurrence. This assumption is supported by the large body of evidence showing that OBI has the highest prevalence precisely in HCV-infected patients [5, 39], and by the in vitro data demonstrating that the HCV "core" protein can strongly inhibit HBV replication and gene expression [77–80]. However, more recent evidence has challenged the existence of interplay between HCV and HBV. Indeed, studies performed in animal models coinfected with HCV and HBV or in hepatoma cells transfected with HCV replicon (instead of single viral proteins) and full-length HBV genome have found no interference between the two viruses [81–85]. Thus, the available data cannot allow any definitive conclusion to be drawn for a role of HCV in the induction of the OBI status. It is known that also individuals positive for human immunodeficiency virus (HIV) frequently show either overt or occult HBV coinfection, but there is no evidence of possible direct effects of HIV on HBV activity or of the existence of any peculiar specific mechanisms leading to OBI occurrence in HIV-infected individuals [86–88]. Other infectious agents potentially capable of interfering with HBV activity include *Schistosoma mansoni*, a parasite that affects more than 200 million people worldwide [89]. Coinfection with HBV and Schistosoma occurs frequently in geographic areas where both agents are endemic [90, 91], and it has been demonstrated that infection with Schistosoma mansoni in HBV transgenic mice induces a strong suppression of HBV replication [92].

Prevalence

The peculiar life cycle of the HBV with its long-term persistence at intrahepatic level regardless of the HBsAg status represents scientific support of the large body of evidence indicating that OBI is a common, worldwide occurrence. Nevertheless, a reliable evaluation of the general prevalence of OBI is at present a very difficult objective to achieve mainly because of the lack of standardized, valid and commercially available assays for its detection, and because the present gold standard for OBI identification (i.e., to test liver DNA extracts by highly sensitive and specific molecular biology approaches such as nested-PCR or real-time PCR) is of course applicable only in the little minority of cases in which a liver specimen is available [1]. In addition, the positivity of circulating anti-HBc antibody—often used as a surrogate marker for OBI identification in HBsAg negative subjects—may be misleading since anti-HBc tests may provide false positive results [1, 93, 94], and also because about 20 % of OBI cases are negative for all HBV serum markers (namely, OBI

seronegative individuals) [1, 5, 39]. Despite the above-mentioned limitations and some discrepancies in the available epidemiological data mainly due to the differences in sensitivity and/or specificity of the methods used in the various studies (reviewed in refs. [5, 95]), there is more than one solid piece of evidence that OBI is a largely world-wide diffused entity with a distribution that may reflect the diffusion of the HBV in the various geographic areas and in the various populations [96–99], and thus with a prevalence that appears to be higher in countries where HBV is endemic and among subjects at high risk of parenterally transmitted infections such as drug addicts and hemophiliacs [100, 101]. Of importance, OBI appears to be highly prevalent in chronically HCV infected individuals, and generally in patients with chronic liver diseases (i.e., alcoholic, cryptogenic, etc.) or with hepatocellular carcinoma [5, 39, 46, 78, 102–105]. In fact, HBV DNA is detectable in about one third of HBsAg-negative HCV carriers in the Mediterranean area, in more than 50 % in Far East Asian countries and in 50 % of US patients of Caucasian origin undergoing liver transplantation for end-stage HCV-related liver disease [39, 95, 106]. This last observation is particularly important also considering that the HBV general prevalence in the Caucasian American population is one of the lowest in the world [107].

Clinical Implications

The vast majority of individuals with OBI will never suffer from any clinical event related to the small amounts of viral genomes segregated in the liver cells. Nevertheless, in some particular circumstances and contexts OBI may acquire a pathogenic role and may become a (co)factor implicated in different clinical conditions that may also have severe sequels (Fig. 13.2). Indeed, since the suppression of viral replication and gene expression typical of the OBI status is a reversible condition, there is no doubt about the possibility that OBI, once transmitted by blood transfusion or liver transplantation from an "occult carrier," may induce a typical, overt hepatitis B in a recipient naive for HBV infection. In analogy, an occult HBV infection may be reverted in an overt infection and reactivated with development of hepatitis B—often acute and severe—in patients undergoing therapeutic immunosuppression. Moreover, growing evidence exists on the possible contribution of OBI to the progression of liver fibrosis and establishment of cirrhosis as well as to the development of hepatocellular carcinoma, this last effect being related to the maintenance in the OBI phase of the mechanisms responsible for the pro-oncogenic properties of the overt, active HBV infection. In this context, however, it has to be taken into account that OBI appears to shape up as a complex scenario, which includes several different clinical/virological conditions quite different from one another. In fact, it is possible to distinguish *seropositive* (anti-HBc and/or anti-HBs positive) and *seronegative* (both anti-HBc and anti-HBs negative) OBI individuals (Fig. 13.3). In seropositive OBI, the HBsAg may have disappeared either very early after the resolution of an acute hepatitis event or after many years of overt carriage, whereas the seronegative OBI cases might have either progressively lost all HBV serum markers or

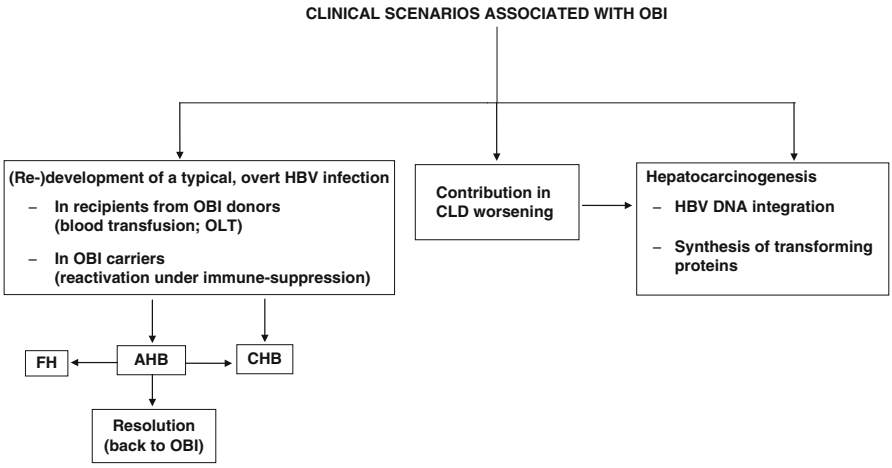

Fig. 13.2 Schematic representation of different conditions associated with the lack of detectable HBsAg in individuals with occult HBV infection (OBI)

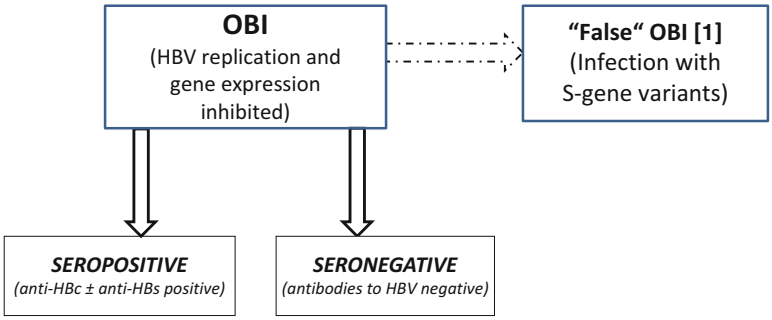

Fig. 13.3 Schematic representation of the possible clinical implications of the occult HBV infection. *OBI* occult HBV infection, *OLT* orthotopic liver transplantation, *CLD* chronic liver disease, *FH* fulminant hepatitis, *AHB* acute hepatitis B, *CHB* chronic hepatitis B

might have been HBV negative since the beginning of the infection. Indeed, one cannot rule out the possibility that each of these conditions may have different roles and/or impacts on the occurrence or outcome of the liver disease.

HBV Transmission from OBI Patients

Transmission Through Blood Transfusion

All blood donations containing HBV DNA are potentially infectious also in the absence of HBsAg. Thus, carriers of occult infection with residual circulation of viral genomes may be a source of HBV transmission in the case of blood

transfusion with the consequent, possible development of typical hepatitis B in the recipients [11, 93, 94, 108]. This possible occurrence was first reported in the late 1970s and then experimentally confirmed in chimpanzees [109–111]. Thus, the high level of alert still maintained in blood banks for identification of OBI positive donors is more than justified. Thanks to this alert and the implementation of progressively more sensitive and specific diagnostic tests, the risk of HBV infection after blood transfusion has dramatically decreased in the last decades, and in fact post-transfusional hepatitis B is now a rare event in the western world. In this context, however, it is important to consider that epidemiological studies based on the most sensitive screening tests for HBV detection [i.e., Nucleic Acid Testing (NAT)] have shown that the frequency of HBV DNA positive cases among HBsAg negative blood donors varies considerably according to the prevalence of the infection in the different geographical areas. Since HBV is highly endemic in many developing countries that have not yet adopted the expensive NAT techniques for blood screening, the persistence of a not negligible risk of HBV transmission by transfusion in the less rich areas of the world is understandable. Schematically, the transfusional transmission of HBV may occur when the donor is an "OBI carrier" in two different situations:

1. The donor has a typical occult HBV infection with wild-type HBV populations suppressed in their replication and gene expression capabilities. In this context, it has to be considered that chronic occult infection is frequently characterized by periods of transient HBV viremia alternating with periods in which the viral DNA is undetectable in the serum [112, 113]. Thus, an "occult HBV carrier" may have a profile of blood infectivity fluctuating over time, although it has to be taken into account that the amount of circulating viruses is usually very low and the amount sufficient to induce an acute hepatitis B event in the recipient remains questionable. Moreover, apart from the viral load of the donor, the possibility of inducing acute hepatitis likely depends on a sum of factors including the amount of plasma transfused and the immuno-competence of the recipient. Nevertheless, the lack of acute hepatitis development does not exclude the possibility that the HBV has been transmitted and infection has occurred, with the consequent theoretical and intriguing possibility that the recipient might in turn become an occult HBV carrier.
2. The donor is infected with S-escape HBV mutant strains. Infection with these genetic variants has also been named "false OBI" since the virus may normally replicate but it synthesizes modified surface proteins that are not identified by the HBsAg diagnostic kits (Fig. 13.3) [1]. This condition appears to be a major cause of the very few, residual cases of HBV transmission by blood transfusion in the most developed countries [93, 94].

Transmission Through Liver Transplantation

OBI transmission may also occur in cases of orthotopic liver transplantation (OLT) and—much less frequently—in cases of kidney, heart and bone marrow transplantation (reviewed in ref. [5]). De novo hepatitis B in OLT HBV naïve recipients

receiving the organ from an OBI donor is a frequent and well-recognized occurrence. It is the clear consequence of the fact that the liver cells are the reservoir of the viral populations, and it largely explains and justifies the anti-HBV prophylaxis [with high doses of anti-HBs immunoglobulin and NUCs inhibiting the HBV reverse-transcriptase] that is generally performed in HBsAg-negative transplanted patients who receive livers from anti-HBc positive donors (HBV transmission from OBI seronegative donors is uncertain and, in any case, very difficult to diagnose). This prophylaxis appears to be very effective in preventing HBV hepatitis in the recipients [114] but it is insufficient to avoid HBV reinfection and the establishment of a new occult infection [115]. In fact, there is clear evidence of OBI occurrence in transplanted individuals who were occulting infected prior to OLT and/or received the new organ from an OBI carrier. In the transplanted liver, viral DNA (including HBV cccDNA) is present and may derive from occult viruses previously infecting the recipient, the donor or even both [116]. An important topic of debate is whether OBI might have any clinical impact in the long-term outcome of OLT patients. In this context, some preliminary evidence suggests a possible involvement of OBI in a faster progression toward cirrhosis of the post-OLT liver disease in patients with HCV infection [117, 118]. Finally, it is appropriate to point out that occult infection also develops in all HBsAg-positive transplanted patients who receive anti-HBV prophylaxis and become HBsAg-negative in the post-OLT period but invariably show the reinfection of the liver [116].

HBV Reactivation in Cases with Occult Infection

As stressed above, an HBV infection enters in the occult phase when the host's defense mechanisms (essentially the mechanisms of immune surveillance) succeed in determining a potent inhibition of viruses that are per se competent in their replication and gene expression capabilities. Thus, all conditions inducing profound changes of the host's immunological status and the interruption of the efficient control of the HBV activities might lead to OBI reactivation with the consequent possible development of a typical acute hepatitis B showing (re-)appearance of HBsAg and even of HBeAg, and with a clinical behavior that is often severe and sometimes fatal for the patient (reviewed in refs. [40, 119–121]. In this context, it is worth mentioning some interesting although anecdotic reports indicating that OBI reactivation might also occur under treatment with histone deacetylase inhibitors, thus suggesting the possibility that also drugs potentially influencing the epigenetic control of the HBV cccDNA minichromosome might cause viral reactivation [122, 123].

HBV reactivation is almost the rule in inactive HBsAg-positive patients undergoing immune-suppression, whereas the frequency with which it occurs in OBI carriers is still undefined. In this context, it has to be considered that OBI individuals may frequently change their HBV serological profile if immuno-compromised. In fact, the anti-HBs antibody—when present—may progressively disappear during immune-suppressive therapy and this occurrence may be followed by HBsAg reappearance that, however, is accompanied by development of clinically evident

acute hepatitis in only a few cases [124–126]. Consequently, OBI reactivation appears to be an event occurring more frequently than usually believed, but it is often clinically silent and the diagnosis might be missed in many cases. Nevertheless, although the incidence of reactivation in individuals with OBI is much lower than in overt HBV carriers, it has considerable importance and represents an every-day challenge in clinical practice because of both the huge number of potential "OBI carriers" (namely, anti-HBc positive individuals) worldwide and the availability of new, potent and efficacious immunological drugs and complex chemotherapy schedules longitudinally administered over several subsequent cycles in different clinical contexts. Indeed, this topic has been discussed in all international guide lines for the management of HBV infection published in the last few years, and it is also included in a recent alert by the FDA directed to physicians of various specialties and concerning the risk of HBV reactivation in patients undergoing anti-CD20 therapies [127]. At present, no reliable marker that helps in predicting HBV reactivation in OBI patients is available. In fact, there are contrasting data on the possibility that patients positive for anti-HBc alone have different risks of OBI reactivation compared to those positive for both anti-HBc and anti-HBs [124, 128, 129] and whether detectable serum HBV DNA at basal time before starting immune-suppressive therapy has any value in predicting the reactivation is also debated [128]. However, on the basis of the literature data, clinical/therapeutic conditions at higher or at lower risk for the occurrence of reactivation have been identified (Table 13.1). Indeed, patients with hematological malignancies (in particular, non-Hodgkin lymphoma, multiple myeloma, myelo-monoblastic acute leukemia, chronic lymphocytic leukemia) have the highest risk of OBI reactivation, especially when treated with schedules including anti-CD20 monoclonal antibody (i.e., Rituximab, Ofatumumab) and, in particular, combinations of Rituximab with Cyclophosphamide, Hydroxydaunorubicin, Oncovin and Predniso(lo)ne, R-CHOP [40, 119, 124, 130–133]. Another category of individuals showing a quite high incidence of OBI reactivation are patients undergoing hematopoietic stem cell transplantation (HSCT) [125, 126]. OBI reactivation appears to be an infrequent—but existing—event in individuals with rheumatologic diseases undergoing treatments including biologics (mainly, anti-CD20 but also anti-TNFα drugs) or with schedules containing high doses of corticosteroids [40, 132–136]. Anecdotic cases of OBI reactivation in patients with HCC undergoing trans-arterial-chemo-embolization as well as in patients with inflammatory bowel diseases under treatment with biological agents have been reported [5], whereas several doubts exist on the real risk of OBI reactivation in patients with solid tumors undergoing chemotherapy [137], and no report exists about OBI reactivation in other categories of patients undergoing treatments with biological drugs (i.e., individuals with psoriasis).

Apart from anti-CD20, a number of other drugs have been reported to be associated with some cases of OBI reactivation (reviewed in refs. [5, 40, 120, 124, 130]): in particular, the anti-CD52 monoclonal antibody Alemtuzumab that is used in onco-hematology therapeutic schedules [138], and the anti-TNFα drugs that are largely utilized for treatment of autoimmune, inflammatory diseases (of note, TNF-alpha is a chemokine able to inhibit HBV replication [139]). Finally, also corticosteroids administered at high doses and for long periods may be involved in OBI

Table 13.1 Schematic representation of conditions exposing at different risk of HBV virological/clinical reactivation in OBI carriers or in recipient from OBI donors

Higher risk
For OBI carriers
– Onco-hematological malignancies under treatment
– R-CHOP (rituximab–cyclophosphamide, adriamycin/doxorubicin, vincristine, prednisone) treatments
For recipients
– Liver transplantation
– Hematopoietic stem cell transplantation
Lower risk
For OBI carriers
– Rheumatological diseases treated with biological agents or high dosage of steroids for prolonged time
– HIV infection
– Inflammatory bowel diseases treated with biologics
– Trans-arterial chemoembolization for treatment of hepatocellular carcinoma
For recipients
– Kidney transplantation
– Bone marrow transplantation
Uncertain risk
For OBI carriers
– Dermatological diseases treated with biologics
– Solid tumors treated with chemotherapy
For recipients
– Organ transplantation different from liver and kidney

reactivation as a possible consequence of both their immune-suppressive effects and their capacity to directly stimulate the HBV replication through the glucocorticoid responsive element present in the viral genome [140].

While prophylactic anti-HBV therapy with NUC inhibitors is a generally adopted practice for the prevention of reactivation in inactive HBsAg-positive carriers undergoing immunosuppressive therapies, the prophylactic antiviral treatment of patients suspected to be OBI positive is still a matter of debate. Indeed, NUCs treatment of onco-hematologic HBsAg negative/anti-HBc positive patients before starting R-CHOP therapy is now quite widely adopted in clinical practice [40]. In all other clinical/therapeutic contexts in which the risk of OBI reactivation is lower, strict surveillance is nevertheless recommended (see also the guidelines for the management of Chronic Hepatitis B licensed by the European Association for the Study of the Liver) and these patients should be followed by alanine-aminotransferase (ALT) and HBV DNA testing and treated with a NUC upon confirmation of HBV reactivation before ALT elevation to prevent hepatitis development [8]. Finally, an additional point worthy of discussion concerns the question of whether HBV reactivation may also occur in patients with seronegative OBI. Of course, this subset of patients is very difficult to identify because of the lack of any marker that helps when the infection is suspected. Indeed, convincing data are available showing that the HBV-specific

T cell response is much weaker in OBI sero-negative than in OBI sero-positive individuals, thus likely insufficient to provoke immune-mediated liver injury [47]. According to this observation one may suppose that OBI reactivation is a phenomenon only occurring in anti-HBV antibody-positive subjects.

Occult HBV Infection and Chronic Liver Disease

An important and widely debated topic is whether occult HBV may favor the progression toward cirrhosis of chronic liver disease (CLD) in HCV-infected patients (as well as in individuals with liver disease of other etiology), as suggested by a quite large body of evidence and confirmed by a recent meta-analysis [5, 39, 141]. Indeed, how OBI may induce (or contribute to) liver injury despite the profound suppression of its replication and gene expression is difficult to explain, and one can only postulate some hypotheses. In this context, it seems important to consider that individuals who have recovered from self-limited acute hepatitis usually show no clinical or biochemical sign of liver damage but, when their liver tissue is examined even several decades after the resolution of the acute hepatitis, HBV genomes are invariably detected together with histological patterns of a mild necroinflammation [142–145]. Moreover, these individuals maintain a very high level of specific anti-HBV cytotoxic T lymphocyte (CTL)-response even many years after clinical recovery and anti-HBs seroconversion, as a possible consequence of the continuous stimulus exerted by the minute amounts of viral proteins that OBI produces [41, 42]. In addition, studies performed on the woodchuck model analyzing liver tissues of these rodents showed that animals that have recovered from acute hepatitis due to WHV show a life-long persistence of an occult infection associated with a mild but persistent liver necroinflammation [146]. Long-term studies evaluating HCV patients with contemporary occult HBV infection have shown that phases with a rise of ALT levels correspond to the reappearance of circulating HBV DNA [112, 113]. Summarizing, all these observations might suggest that patients with OBI show transient phases of viral reactivation over time that is promptly controlled by the CTL-response, although a modest but histologically evident degree of liver damage persists.

A recent long-term observational cohort study evaluating the clinical outcome of a large series of chronic hepatitis C patients tested for OBI by liver DNA analysis in the 1990s and followed up for a median time of 11 years showed that OBI is significantly associated with both development of HCC (see below) and the most severe evolution of the CLD (i.e., decompensated cirrhosis), and finally that chronic HCV patients with OBI have a significantly increased risk of liver-related death compared to OBI-negative patients. Notably, the negative effects of OBI disappeared in patients therapeutically cured from hepatitis C [147].

Altogether, these data seem to confirm the hypothesis that—at least in immune-competent individuals—the occult infection is in itself innocuous, being unable to provoke a clinically significant liver injury, but if other causative agents of liver injury co-exist (i.e., HCV infection, alcohol abuse, etc.) it might be a factor making the course of the liver disease worse [148].

A further point that has to be considered is that part of the patients with productive HBV infection and classic chronic hepatitis B, after years or decades of HBsAg carriage, may show a progressive reduction of viral replication and amount of serum HBsAg that may even disappear over time with consequent development of OBI. However, if cirrhosis had already been established during the overt phase of the infection it obviously persists also in the occult phase, and, importantly, the risk of HCC development persists although reduced in comparison with cases of long-lasting HBsAg positive status [149–152].

Occult HBV Infection and HCC

HBV is a well-recognized oncogenic virus and one of the main etiologic agents of HCC worldwide. Much evidence indicates that HBV may maintain its pro-oncogenic propensity also when it is in the occult phase of the infection [153, 154]. Subjects with chronic hepatitis C appear to be particularly prone to HCC development in cases with concomitant OBI [155–157], as also confirmed by the above-mentioned long-term observational cohort study that evaluated the clinical outcome of chronic HCV patients according to their OBI status [147]. Moreover, a recent meta-analysis subsequently confirmed that OBI is an important risk factor for HCC development not only in HCV-infected individuals but also in patients with CLD unrelated to viral infection [158]. Indeed, among HCV-negative patients OBI seems to exert its tumorigenic effect in individuals with genetic and alcoholic diseases as well as in individuals with cryptogenic CLD [44, 159–161]. In this context, a recent population-based cohort study, conducted for more than two decades on Taiwanese mothers screened for HBV infection at each delivery, should be mentioned. This study showed that HCC occurrence was significantly associated with the persistence of the HBsAg-positive status, but among the HBsAg-negative mothers those who underwent HBsAg seroclearance during the follow-up had a significantly higher risk of HCC development compared to women never exposed to HBV [162]. Thus, this study shows that HBV maintains its hepatocarcinogenetic role after becoming occult even in the women that are known to be much less prone to develop liver cancer than men. Interestingly, a further recent study indicates that individuals undergone HBsAg seroclearence have a risk of HCC development comparable to that of subjects with persisting HBsAg positivity but with undetectable serum HBV DNA [163]. A final important note concerns the studies performed in woodchucks and in ground squirrels. Both these rodents are susceptible to hepadnavirus infections and have a high risk of developing HCC also after the apparent clearance of the hepatitis virus with disappearance of the viral surface antigen and seroconversion to the corresponding antibody but, invariably, with the long-term persistence of viral DNA at intrahepatic level [164, 165].

Summarizing, large parts of the available data indicate that OBI is a potential pro-oncogenic condition. Although the pathogenesis of the OBI-induced hepatocarcinogenesis still has to be mostly elucidated, evidence exists that helps to delineate the mechanisms through which the occult HBV might contribute to hepatocyte

transformation. Indeed, it is generally accepted that an overt and active HBV infection may exert its pro-oncogenic role both indirectly (by inducing a chronic state of necroinflammatory liver injury that may progress through cirrhosis to HCC) and directly [by the synthesis of viral proteins (i.e., X protein, truncated preS/S proteins) provided with pro-oncogenic properties and by the propensity of the viral DNA to integrate into the host's genome] [153, 154]. OBI might maintain both indirect and direct tumorigenic potentialities. As reported above, in fact, it may induce a very mild but persistent necro-inflammation of the liver that—when another concomitant cause of liver injury is present—may contribute to the development of cirrhosis that is the most important predisposing factor of liver cancer. In addition, HBV DNA integration may be present in the occult infection, and low levels synthesis of viral proteins—including X and mutated preS/S proteins—may persist in the OBI phase.

Conclusion and Perspective

OBI is a fascinating and intriguing topic of viral hepatitis field, and learning about it appears to be of great importance for an overall understanding of HBV infection. In recent years a large number of studies have made it possible to disclose several of its virological aspects, to show its worldwide diffusion and to reveal its possible implication in various clinical contexts. The molecular basis of OBI is related to the long-term persistence of HBVcccDNA in the nuclei of the liver cells despite the absence of viremia and the HBsAg negativity, and indeed OBI appears to be a phase in the natural history of chronic HBV infection. The mechanisms determining OBI status have still to be mostly clarified, but it is evident that host defense mechanisms play an essential role in its induction by suppressing the viral replication and gene expression. Occult HBV infection is a well-known danger for human health in terms of risk of viral reactivation in conditions of immunosuppression as well as of transmission of the infection during liver transplantation. Increasing evidence also indicates that it may favor the progression toward cirrhosis of chronic liver diseases related to different etiologies and above all that it maintains most of the pro-oncogenic properties of overt HBV infection. Diagnosis of OBI currently relies on non-standardized techniques and can be performed only in highly specialized laboratories. Thus, the development in the near future of valid and commercially available assays allowing the detection of OBI in all cases in which its presence might be a clinical risk appears to be of great importance and the true challenge in this field of research.

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