Pathogenetic and antiviral immune responses against hepatitis B virus

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Hepatitis B virus (HBV) is a noncytopathic virus that causes liver disease of variable duration and severity. It is widely assumed that during HBV infection the host immune response is responsible for both hepatocellular damage and viral clearance. Whereas there is considerable evidence that the innate immune response does not play a significant role in these processes, the adaptive immune response, particularly virus-specific cytotoxic T lymphocytes (CTLs), seems to contribute to nearly all of the liver injury associated with HBV infection. By killing infected cells and producing antiviral cytokines capable of purging HBV from viable hepatocytes, CTLs are also thought to eliminate the virus. Although liver damage is initiated and mediated by the CTLs, antigen-nonspecific inflammatory cells can worsen CTL-induced immunopathology and platelets may facilitate the accumulation of CTLs in the liver. The mechanisms responsible for disease pathogenesis and viral clearance during HBV infection are the subject of this review.

Keywords: chemokines, cytokines, cytotoxic T cells, hepatitis B virus, platelets, viral hepatitis

future medicine

Hepatitis B virus (HBV), the prototype member of the *Hepadnaviridae* family, is an enveloped virus with a circular, double-stranded DNA genome that causes acute and chronic necroinflammatory liver disease [1,2]. Over 95% of acutely infected adults completely and spontaneously recover from infection, whereas over 90% of transmitted infections become persistent [1,2]. Chronic HBV infection is associated with varying degrees of liver disease and often progresses to the development of life-threatening complications, such as cirrhosis and hepatocellular carcinoma (HCC) [1,2]. On a worldwide basis, over 350 million people are infected chronically by HBV and approximately 1 million of these die each year from the complications of chronic infection [1,2]. As many of these patients do not have a sustained response to currently available therapies (nucleoside analogs and interferon [IFN]-α) [1,2], it is obviously very important to improve our understanding of HBV pathogenesis in order to develop improved treatments.

Studies of HBV pathogenesis have been hampered by the fact that the host range of this virus is restricted to humans and chimpanzees and also owing to the lack of cell-culture systems and small-animal models that are readily susceptible to infection. Over the last few years, however, many questions pertaining to the immune-mediated mechanisms responsible for disease pathogenesis and viral clearance during HBV infection have been addressed successfully and are described in this review.

Innate immune responses to HBV

As viruses adhere to, enter and replicate within target cells, early innate defense mechanisms are triggered rapidly by the host in order to contain viral spread [3–5]. These defense mechanisms comprise the induction of apoptosis by the virus [3], the production of antiviral cytokines, such as IFN-αβ, by the infected cells [4] and the activation of effector functions of cellular components of the innate immune system, such as natural killer (NK) and NK T cells [5]. Current understanding of these early processes during HBV infection reflects largely what has been learned in chimpanzees, since these animals can be studied from the onset of infection through the course of the associated disease.

Evidence that HBV can trigger apoptosis is lacking since, during the early phase of HBV infection in chimpanzees (i.e., before virus-specific T cells enter the liver), there is no histological or biochemical evidence of hepatocellular injury [6,7]. In addition, HBV is able to replicate at high levels in the liver of both patients and transgenic mice noncytopathically, when cellular immune responses are deficient or pharmacologically suppressed [1,2,8]. Evidence that HBV induces infected cells to produce IFN-αβ is also lacking. Indeed, global gene expression profiling, performed on liver RNA samples from HBV-infected chimpanzees, indicates that no IFN-αβ or IFN-αβ-responsive genes are induced in the organ before adaptive immune responses are triggered, even at a time when all of the

hepatocytes are infected [9]. Although activation of NK and NK T cell, or the engagement of Toll-like receptor, have been shown to inhibit viral replication in HBV transgenic mice [10–12], there is still no evidence that these cells or pathways of the innate immune system play a role in disease pathogenesis or viral clearance during the initial phase of HBV infection. Indeed, activated NK and NK T cells are an abundant source of IFN-γ [5] and IFN-γ or IFN-γ-inducible genes have not been detected in the liver of chimpanzees during non-cytopathic spread of HBV throughout the liver [6,7,9]. These results collectively indicate that early innate defense mechanisms contribute to neither the pathogenesis of liver injury nor to viral clearance, and that HBV spreads and remains undetected until the adaptive immune response enters the liver.

Adaptive immune responses

Virus-specific $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) contribute to tissue damage and participate in viral clearance, either by killing infected cells or by producing soluble factors, such as cytokines and chemokines, which contribute to the inflammatory process and/or inhibit viral replication [1,13]. T-cell-derived cytokines and chemokines also play a role in the shaping of antiviral antibody responses that take part in viral clearance, mainly by blocking virus entry into susceptible cells and by removing infectious virions from the circulation.

T helper (Th) cells are usually primed within secondary lymphoid organs by antigen-presenting cells (APCs) that have internalized soluble viral antigens. Based on their central role as regulators of the immune response in other viral infections [14], it is likely that they contribute to HBV infection control mainly by facilitating the induction and maintenance of virus-specific CTLs, as has been suggested for HCV [15]. In keeping with this, relatively vigorous HBV-specific Th responses are always associated with quantitatively and qualitatively significant CTL responses in both humans and chimpanzees that resolve HBV infection [1]. Despite the fact that Th cells have been shown to have cytolytic activity *in vitro* and possibly *in vivo* as well [16], it is unlikely that these cells play an important pathogenetic role in HBV infection. Indeed, recent observations in an acutely HBV-infected chimpanzee in which $CD4^+$ T cells were depleted at the peak of infection indicated that the liver disease in this animal was comparable to that detected in immunologically unmanipulated controls [7].

In contrast to Th cells, priming of CTLs normally requires the processing of viral proteins that are either endogenously produced or phagocytosed by professional APCs [17,18]. For viruses, such as HBV, that do not infect professional APCs much, tissue-derived dendritic cells (DCs) that have internalized apoptotic virus-infected cells and debris are expected to migrate to the regional lymph nodes to permit T-cell priming [17,18]. Clonally expanded CTLs can leave the lymph nodes, enter the bloodstream, reach the infected organ, exit the bloodstream and enter the parenchyma to recognize viral antigen and perform their effector functions. In most viral diseases, CTLs are usually detected at sites of infection within 1 week of exposure [19], whereas the initial entry of T cells into the liver of chimpanzees acutely infected with HBV does not occur until a few months post-infection [6,7]. Since virus-specific CTLs are already measurable in the peripheral blood of these animals by weeks 2–3 post-infection [7], these results suggest that processed viral antigen is absent from the surface of infected hepatocytes during active viral replication, an event that may be facilitated by the fact that major histocompatibility complex (MHC) class I expression is minimal on hepatocytes, except in the context of an inflammatory response [20].

The notion that virus-specific CTLs play a fundamental role in the pathogenesis of liver disease and viral clearance during HBV infection is supported by the following data. First, the beginning of liver injury corresponds kinetically with the influx of virus-specific CTLs into the liver of chimpanzees infected by HBV [6,7] and depletion of these cells at the peak of viremia delays the onset of biochemical, histological and clinical evidence of viral hepatitis as well as viral clearance [7]. Second, the association between the magnitude of virus-specific CTL responses, liver disease severity and viral clearance has been reported not only in infected chimpanzees, but also in many studies in patients infected with HBV [1]. Third, the adoptive transfer of HBV-specific CTL lines and clones into immunologically tolerant HBV transgenic mice triggers a necroinflammatory liver disease that shares the same histological features of acute viral hepatitis in humans and results in the inhibition of

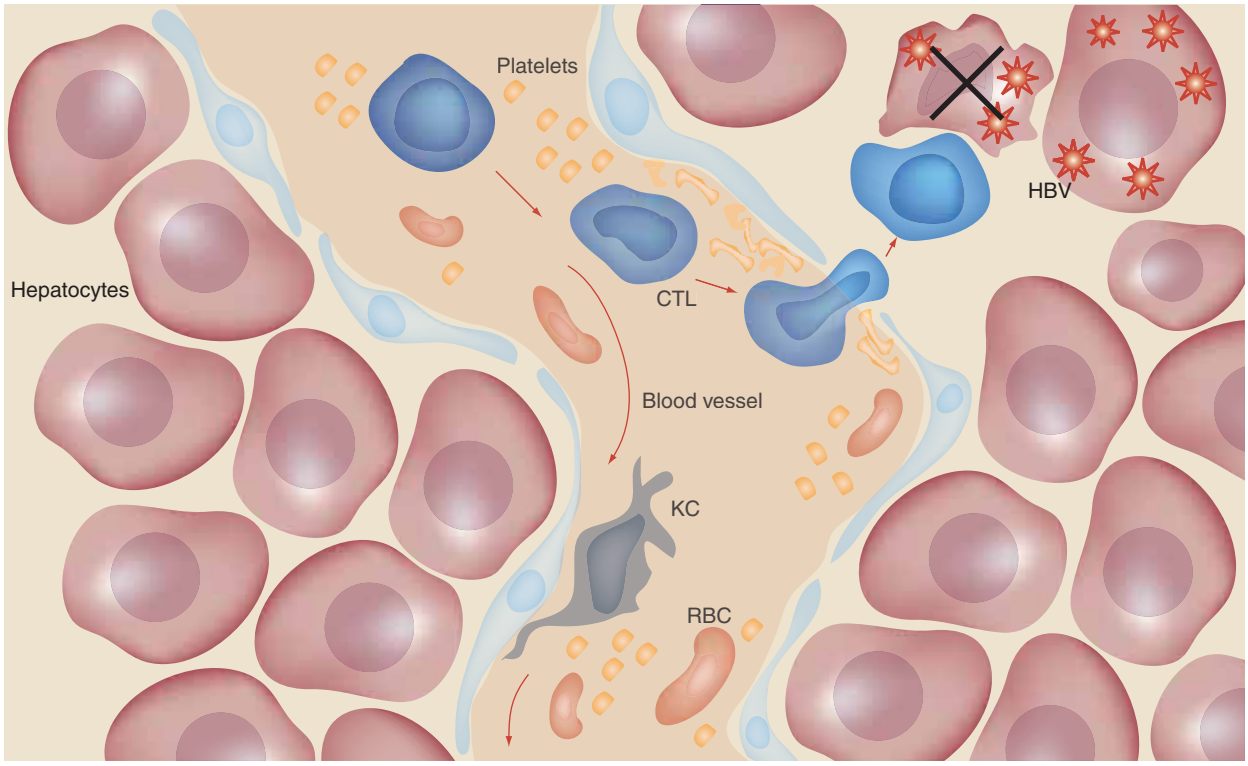
HBV replication [21,22]. The availability of a small-animal model with a well defined immune system (i.e., the HBV transgenic mouse) has allowed definitive analysis of many of the immunological mechanisms involved in HBV pathogenesis. In the course of those studies, several other previously unknown aspects of viral pathogenesis have been elucidated and are summarized below.

The way in which CTLs leave the hepatic circulation and enter the liver parenchyma is poorly understood; however, recent studies have shed new light on this process. These studies showed that following the transfer of virus-specific CTLs into HBV transgenic mice, platelets are readily detectable within necroinflammatory foci of the liver, and their selective depletion ameliorates the severity of the disease [23]. The profound reduction in liver disease severity observed in thrombocytopenic animals is associated with a nearly proportional reduction in the intrahepatic accumulation of antigen-specific CTLs, both of which are restored upon reconstitution with platelets capable of becoming activated *in vivo*. The mechanism through which activated platelets may facilitate the intrahepatic accumulation of CTLs is suggested by *in vitro* findings, indicating that under the low shear-rate flow conditions likely to occur in the venous circulation of the liver, virus-specific CTLs interact tightly with platelet aggregates [23]. Thus, one can envisage that an initial, immune-induced inflammatory response results in changes in the vessel wall that promote platelet adhesion and activation, which in turn favor the exit of virus-specific CTLs from the bloodstream and their accumulation within the liver parenchyma (Figure 1). This process is probably facilitated by specific interactions between CTLs and platelets and, in this regard, it is worth mentioning that T lymphocytes can interact with E- and P-selectin [24,25] and activated platelets present abundant membrane expression of P-selectin [26]. Platelets could also contribute to liver damage by amplifying antigen-nonspecific inflammatory responses. The formation of fibrin clots in extravascular spaces is one of the hallmarks of inflammation and, indeed, the livers of CTL-injected animals showed abundant fibrin deposition. Activated platelets are also known to express procoagulant activities that lead to fibrin formation [27,28], and the localization of platelet aggregates and fibrin within hepatic necroinflammatory foci suggests the possibility that platelets could also contribute to tissue damage by inducing clotting. It can be deduced convincingly that this is not the case from the demonstration that CTL-induced liver damage progresses unchanged even when fibrin deposition is completely prevented by anticoagulant therapy [23]. Consequently, the pathogenic role of platelets in CTL-induced liver injury may be primarily played in support of immune-mediated responses, rather that as part of nonspecific inflammatory reactions.

As CTLs reach the liver parenchyma, the first step in the disease process is antigen recognition by these cells, which rapidly induces hepatocellular apoptosis (Figure 2) [21]. The fact that, upon transfer into HBV transgenic mice, HBV-specific CTL clones that are genetically deficient in either FasL or perforin do not induce hepatocellular apoptosis, indicates that both the perforin and Fas death pathways must be activated simultaneously for CTLs to be cytopathic in the liver [29]. The initial apoptotic process, however, involves a relatively small number of hepatocytes [21] and as time progresses, many host-derived inflammatory cells are recruited into the liver, thereby contributing to the formation of necroinflammatory foci scattered throughout the liver parenchyma, in which apoptotic hepatocytes and virus-specific CTLs are outnumbered by host-derived mononuclear and polymorphonuclear inflammatory cells (Figure 2) [21,30,31]. As mentioned earlier, the histological appearance of these lesions is very similar to classical viral hepatitis in humans.

Recruitment of host-derived antigen nonspecific inflammatory cells into the liver is a process that is associated with the intrahepatic production of chemokines and it is likely to contribute to the pathogenesis of liver disease. Indeed, the authors demonstrated that blocking the chemokines CXCL9 and CXCL10 reduces the intrahepatic recruitment of host-derived mononuclear cells and the severity of CTL-induced liver disease [30]. The authors also showed that CXCL9 and CXCL10 are induced rapidly and strongly in the liver after CTL transfer and the transferred CTLs produce neither chemokine; rather, they activate (via the secretion of IFN-γ) hepatocytes and nonparenchymal cells of the liver to produce them [30]. The association of reduced liver disease with reduced recruitment of antigen-nonspecific mononuclear cells implies that these cells can amplify the liver damage

Figure 1. Platelets facilitate the accumulation of CTLs at sites of infection.



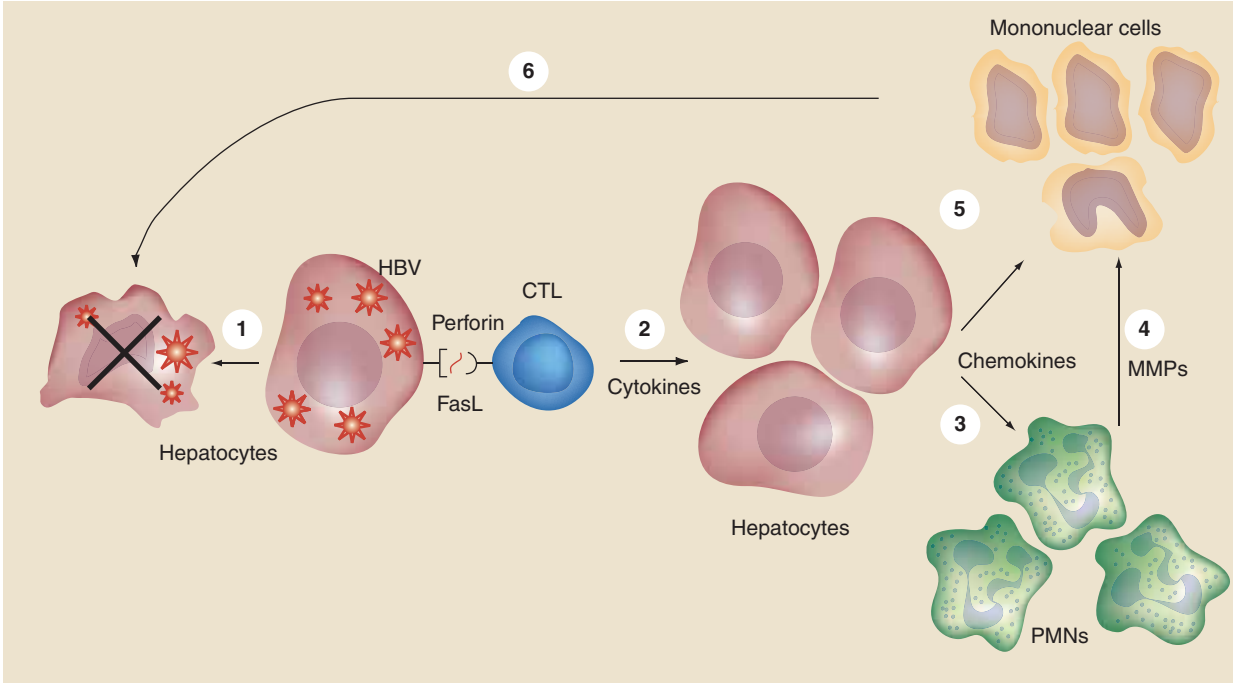
Inflammation-induced changes of the vessel wall may promote platelet adhesion and activation, which in turn favor the exit of virus-specific CTLs from the bloodstream and their accumulation within the liver parenchyma where HBV replicates.
CTL: Cytotoxic T lymphocyte; HBV: Hepatitis B virus; KC: Kupffer cell; RBC: Red blood cell.

initiated by antigen-specific CTLs. Similar mechanisms may contribute to the pathogenesis of viral hepatitis in humans, where, similarly to our system, the number of HBV-specific T cells detected in the liver is outnumbered by recruited nonvirus-specific T cells [32,33] and other inflammatory cells [34].

Recent studies have also demonstrated that depletion of Gr-1$^+$ cells (Gr-1 is an antigen highly expressed by polymorphonuclear neutrophils [PMNs]) abolishes the intrahepatic recruitment of all antigen nonspecific Gr-1$^-$ mononuclear cells (NK and NK T cells, T and B lymphocytes, monocytes, macrophages and DCs), despite the strong induction of chemokine gene expression [35]. This suggests that, in addition to chemokine expression, other CTL-induced functions are necessary for mononuclear cell recruitment to occur. These functions may include the release of the matrix-degrading metalloproteinases (MMPs) by PMNs, which are known to produce high levels of collagenases (such as MMP-8 and neutrophil collagenase) and gelatinases (such as MMP-9 and gelatinase B) [36]. Since MMPs are involved in the remodeling of the extracellular matrix [36], it is possible that these enzymes may facilitate leukocyte trafficking through endothelial barriers and solid organs. In keeping with this, the authors demonstrated that, following CTL transfer, MMP-8 and -9 are not induced in anti-Gr-1-treated HBV transgenic mice and their functional inhibition *in vivo* reduces the CTL-induced recruitment of antigen nonspecific mononuclear cells into the liver and the attending liver disease [37]. The data are compatible with the hypothesis that PMNs represent the first cell type to be recruited into the liver following antigen recognition by CTLs (Figure 2). According to this hypothesis, the production of MMPs by PMNs could remodel the extracellular matrix and facilitate the trafficking of mononuclear cells through the endothelial barrier and into the liver parenchyma in response to their own chemoattractants.

The HBV transgenic mouse model has also been instrumental in showing that the antiviral potential of virus-specific CTLs is mediated primarily by noncytolytic mechanisms involving

Figure 2. Role of CTLs in liver disease and viral clearance.



Following antigen recognition, HBV-specific CTLs kill a small number of hepatocytes (1) via FasL- and perforin-mediated pathways, and produce antiviral cytokines (2) that inhibit HBV replication noncytopathically in a greater number of cells. The same cytokines can activate parenchymal and nonparenchymal cells of the liver to produce chemokines (3) that recruit antigen-nonspecific PMN cells into the organ. Production of MMPs by these cells (4) in addition to chemokine induction (5) contributes to the migration of antigen-nonspecific mononuclear cells (i.e., natural killer cells, T cells and macrophages) into the liver and the amplification of the liver disease initiated by the CTLs (6).
CTL: Cytotoxic T lymphocyte; HBV: Hepatitis B virus; MMP: Matrix-degrading metalloproteinase; PMN: Polymorphonuclear neutrophil.

the local production of high amounts of IFN-γ early after antigen recognition [13,22,38,39] (Figure 2). Indeed, it has been reported that IFN-γ prevents the assembly of replication-competent HBV RNA-containing capsids in the hepatocyte [40], in a proteasome- [41] and kinase-dependent [42] manner. During this remarkable process, the viral nucleocapsids disappear from the cytoplasm of the hepatocytes [22,40] and the viral RNAs are destabilized by a SSB/La-dependent mechanism in the nucleus [43–45], yet the hepatocytes remain perfectly healthy [22,44,46]. One might predict from this that HBV-nonspecific inflammatory responses of the liver could facilitate the clearance of HBV if they induce the local production of antiviral cytokines, such as IFN-γ, to which HBV is susceptible. Precisely these events have been shown to occur in the HBV transgenic mice during unrelated hepatotropic infections of the liver, which include lymphocytic choriomeningitis virus (LCMV) [38,47], adenovirus [38,48], mouse cytomegalovirus (MCMV) [48], malaria [49] or schistosoma [50], or after administration of recombinant murine interleukin (IL)-12 [51], a cytokine produced by APCs that has the ability to induce IFN-γ secretion by T, NK and NK T cells. Along these lines, it has also been shown that a single injection of α-galactosylceramide (αGalCer), a glycolipid antigen presented to $V\alpha14^+$, $NK1.1^+$ T cells by the nonclassical major histocompatibility complex (MHC) class I-like molecule CD1d, inhibits HBV replication by activating NK T cells directly to produce IFN-γ in the liver [11,12]. The notion that IFN-γ produced by activated CTLs can play a direct role in viral clearance was also corroborated by studies in chimpanzees acutely infected with HBV [6,7]. Indeed, it was shown in these animals that most of the viral DNA disappeared from the liver before the peak of liver disease and concomitant with the initial intrahepatic appearance of IFN-γ [6,7]. Moreover, neither intrahepatic IFN-γ induction nor viral clearance occurred in HBV-infected chimpanzees in which CTLs were depleted at the peak of infection [7].

As mentioned earlier, neutralizing antibodies can interfere with viral entry into susceptible cells and thus limit the extent of infection [52].

To date, cell-culture systems capable of being productively infected by HBV are lacking and, thus, the kinetics and function of neutralizing antibodies in the resolution or prevention of HBV is still poorly understood. Evidence that antibodies with neutralizing activity emerge following a self-limited HBV infection is supported by the observation that chimpanzees that resolved a previous infection are completely protected from rechallenge [1]. The appearance of neutralizing antibodies, however, is thought to occur relatively late after HBV exposure and, thus, it is unlikely to play a role in the early phase of viral clearance during acute infection [1]. The evidence that neutralizing antibodies can control long-term noncytopathic infections that are not completely cleared [53], coupled with the observation that complete viral clearance (viral sterilization) following clinical recovery from HBV infection may never occur [54,55], suggest that a sustained neutralizing antibody response may prevent the re-emergence of HBV in patients that have resolved the infection.

Conclusions

Our comprehension of the pathogenesis of HBV infection has advanced significantly in recent years, particularly owing to the experimental use of chimpanzees and transgenic mice. It is becoming increasingly apparent that HBV replicates noncytopathically within the hepatocyte, and that the innate immune response contributes to neither liver disease nor viral clearance. By contrast, the adaptive immune response, mainly the virus-specific CTL response, plays a crucial role in both. Furthermore, recent studies indicate that antigen-nonspecific inflammatory cells enhance CTL-induced immunopathology in the liver, and that platelets facilitate the intrahepatic accumulation of CTLs. Regardless of these accomplishments, however, many questions pertaining to HBV immunobiology and pathogenesis remain to be answered. Future work intended to address these questions will not only expand our current knowledge of host–virus relations that determine the pathogenesis and outcome of infection, but they may also lead to the discovery of new approaches for the treatment of chronic HBV infection and its life-threatening complications.

Future perspective

Listed below is a series of unresolved issues pertaining to HBV immunobiology and pathogenesis. In our opinion, they represent some of the most challenging and important areas of HBV research and should be the focus of future scientific efforts.

- How does HBV recognize and enter the hepatocyte?
- When and where does T- and B-cell priming occur (lymph nodes versus liver) during HBV infection?
- Why does it take so long for virus-specific T cells to accumulate in the liver, recognize antigen and initiate pathogenetic and antiviral activities?
- What are the pathogenetic mechanisms whereby antigen-nonspecific cells amplify CTL-induced liver damage?
- What are the molecular mechanisms whereby platelets facilitate CTL homing?
- What is the relative contribution of cytopathic versus noncytopathic mechanisms in viral clearance?
- Which intracellular genes are responsible for the antiviral activity of CTL-derived cytokines?
- What are the viral targets of such antiviral genes and can HBV mutate them?
- What is the phenotypic and functional evolution of T- and B-cell responses?
- What is the duration of each response and its role in the outcome of infection?
- What are the virological and immunological mechanisms responsible for primary or secondary T-cell unresponsiveness in chronically infected patients?

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Executive summary

Epidemiology

- Over 350 million people are chronically infected with hepatitis B virus (HBV).
- Approximately 1 million people die each year from HBV-related complications.

Natural history

- Most acutely infected adults clear the infection, whereas most neonatally transmitted infection becomes persistent.

Innate immune responses

- HBV replicates noncytopathically within hepatocytes.
- HBV does not induce interferon (IFN)-$\alpha\beta$ or IFN-$\alpha\beta$ responsive genes.
- Innate immune responses do not contribute to disease pathogenesis or viral clearance.

Adaptive immune responses

- Virus-specific cytotoxic T lymphocytes (CTLs) play a fundamental role in viral clearance and liver disease.
- Platelets enhance the intrahepatic accumulation of virus-specific CTLs.
- Antigen-nonspecific inflammatory cells can amplify CTL-induced liver damage.
- Virus-specific CTLs can inhibit HBV replication noncytopathically.

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