Rous-Whipple Award

The Rous-Whipple Award was established by the American Society for Investigative Pathology to recognize a career of outstanding scientific contribution.

The 1999 recipient of the Rous-Whipple Award, Francis V. Chisari, delivered a lecture entitled “Viruses, Immunity, and Cancer: Lessons from Hepatitis B” after accepting the award on Tuesday, April 20, 1999 in Washington, D.C., at the annual meeting of the American Society for Investigative Pathology.

Rous-Whipple Award Lecture

Viruses, Immunity, and Cancer: Lessons from Hepatitis B

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The host-virus interactions that determine the outcome of viral infections have fascinated me ever since medical school. My first opportunity to actively pursue this interest came when I was lucky enough to be part of a team at the National Institutes of Health that transmitted the hepatitis B virus (HBV) to chimpanzees.¹ Thus began a longstanding interest in the immunobiology of persistent human viral infections, especially those that infect the liver. I am greatly honored that this work has been recognized by the Rous-Whipple Award this year, giving me this opportunity to describe our current state of understanding of these infections.

The Infection

As indicated in Figure 1, HBV is a noncytopathic, enveloped, double-stranded DNA virus that causes acute and chronic hepatitis and hepatocellular carcinoma (HCC). HBV is transmitted sexually, parenterally, and from mother to infant at birth, like human immunodeficiency virus (HIV). Most perinatal HBV infections become persistent, presumably due to the failure to mount an effective immune response. In contrast, most adult onset HBV infections resolve, presumably due to the polyclonal, multispecific humoral and cellular immune response that the patients produce against the viral proteins.² More than 2 billion people alive today have been infected by HBV, and more than 350 million people are chronically infected, and these individuals have a 100-fold increased risk of developing HCC.³ Accordingly, HBV causes approximately 1 million deaths each year worldwide. In the United States alone, nearly 300,000 new infections occur annually, more than 1 million people are chronically infected, and more than 5000 of them die each year from cirrhosis and HCC.

Viral hepatitis is a necroinflammatory liver disease of variable severity. Most studies suggest that the hepatitis viruses are not directly cytopathic, or at least not highly cytopathic, for the infected hepatocyte.^{4–6} Since the disease spectrum associated with these viruses is extraor-

dinarily variable, the host response to these viruses must play a critical role in the pathogenesis of the associated diseases. Indeed, based on fairly extensive studies of HBV pathogenesis in man and animal models, there is considerable evidence that viral hepatitis is initiated by an antigen-specific antiviral cellular immune response. Although clearance of most virus infections is widely thought to reflect the killing of infected cells by virus-specific T cells, recent data also suggest that noncytolytic intracellular viral inactivation by certain inflammatory cytokines released by activated lymphomononuclear cells may play an important role in the clearance of at least some of these viruses from the infected cell. This appears to be true for HBV, and much of the evidence for this notion is described below.

The Virus and Its Life Cycle

The infectious 42-nm virion is an enveloped nucleocapsid containing the viral polymerase bound to an incomplete, open circular DNA genome, which consists of a full length 3.2-kb minus strand and an incomplete plus strand (Figure 2). The mechanism of viral entry is not known, although attachment is thought to be mediated by the interaction of the preS(1) region of the large envelope polypeptide with one or more currently undefined hepatocyte receptor(s).^{7–12} After entry and presumptive uncoating (Figure 3), viral plus strand DNA synthesis is completed and the nucleocapsid particle delivers the viral genome to the nucleus. Recent evidence from our laboratory indicates that cytoplasmic nucleocapsid particles do not enter the nucleus.¹³ This suggests that the capsids are arrested at the nuclear membrane and release the viral genome into the nucleus where DNA repair enzymes process and join the viral minus and plus

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Hepatitis B Virus

- **Hepatotropic, noncytopathic, 3.2 kb circular DNA**
- **Acute, chronic hepatitis, hepatocellular carcinoma**
- **Over 2 billion people have been infected**
- **Over 350 million people are chronically infected**
- **100-fold increased risk (40% lifetime risk) of HCC**
- **Over 1 million deaths each year**

Figure 1. The infection.

strands, yielding the 3.2-kb covalently closed circular (ccc) DNA molecule that serves as the viral transcriptional template.¹⁴

The viral genome is organized into 4 transcription units (Figure 2) controlled by 4 independent promoters and a single common polyadenylation signal, yielding 4 extensively overlapping viral RNAs (3.5, 2.4, 2.1, and 0.7 kb in length) that are exported into the cytoplasm where the viral proteins are translated and viral particle assembly and genome replication occurs. The 3.5-kb transcript

HBV MAP

Figure 2. HBV map. The partially double-stranded 3.2-kb open circular genome present in circulating virions is shown at the center. The genome contains the core (C), pre-S (PS), HBs (S), and HBx (X) promoters shown inside round icons as indicated, two enhancers (En I and En II) shown as shaded regions and a single polyadenylation signal (Poly A) resulting in the production of four extensively overlapping transcripts that are 3.5 kb, 2.4 kb, 2.1 kb, and 0.7 kb in length. The 3.5-kb RNA is translated to produce the viral capsid (core) and secreted precore proteins and the polymerase (pol) protein which has reverse transcriptase, RNase H, and DNA polymerase activity. The 3.5-kb RNA is also the viral pregenomic RNA that is packaged with the polymerase protein inside of capsid particles in the cytoplasm where viral replication occurs. The 2.4-kb transcript is translated to produce the large envelope protein, whereas the 2.1-kb transcript is heterogeneous at its 5' end with species that flank the translational start site of the middle envelope protein, such that the shorter species produce the major (most abundant) envelope protein. The 0.7-kb transcript is translated to produce the X protein, which transcriptionally transactivates the viral promoters and several cellular RNAs as well.

produces the polymerase, core, and precore proteins and serves as the pregenomic RNA template that is reverse transcribed as the first step in viral genome replication (see below). The polymerase protein contains a viral RNA packaging signal and performs multiple enzymatic functions including reverse transcriptase, DNA polymerase, and RNase H activity that are essential for viral replication.¹⁵ The core protein, known as hepatitis B core antigen (HBcAg), rapidly forms homodimers that self-assemble into capsid particles¹⁶ in the cytoplasm and the nucleus of the hepatocyte. The intranuclear capsid particles appear to be empty, and their role in the viral life cycle is not understood.¹⁷ The cytoplasmic capsids are true nucleocapsids; ie, they contain the pregenomic viral RNA and the polymerase protein, and it is within these particles that viral genome replication occurs.

HBV replication involves reverse transcription of the RNA pregenome to produce minus strand DNA. The minus strands then serve as the template for viral plus strand DNA synthesis, resulting in an encapsidated double-stranded open circular DNA genome that either recycles back to the nucleus to amplify the pool of cccDNA or becomes enveloped by the viral envelope proteins, buds into the endoplasmic reticulum (ER), and is secreted via the Golgi apparatus into the blood, where it can spread to other hepatocytes.^{14,18–25} The nascent precore protein contains the entire core protein plus a leader sequence that directs it to the ER, where it undergoes limited proteolysis and is secreted into the plasma as hepatitis B e antigen (HBeAg). Its role in the viral life cycle is poorly understood, although it may have tolerogenic properties that would favor viral persistence,^{26,27} and it appears to be able to modulate nucleocapsid stability, and therefore replication, by forming heterodimers with the core protein.^{28–30}

The 2.4- and 2.1-kb transcripts produce the large, middle, and small envelope proteins. The 3 HBV envelope proteins share common carboxy termini and display progressive amino terminal extensions. The small envelope protein contains the hepatitis B surface antigen (HBsAg). The middle envelope protein contains the entire small envelope protein plus an amino-terminal extension in which is located the pre-S(2) antigen. The large envelope protein contains an additional N-terminal extension that defines the pre-S(1) antigen. The envelope proteins are cotranslationally inserted into the ER membrane where they aggregate, bud into the ER lumen, and are secreted by the cell, either as 22-nm subviral envelope particles or as 42-nm infectious virions if they have enveloped the viral nucleocapsids before budding. When the large envelope protein is overexpressed, it forms long branching filamentous particles that accumulate in the ER and are not secreted, causing the ER to become hyperplastic. This gives the cell a "ground glass" appearance histologically³¹ and makes the cell hypersensitive to the cytopathic effects of interferon γ (IFN- γ),³² as will be described below. The 0.7-kb transcript produces the X protein, which has transcriptional transactivating potential³³ and has been shown to be required to initiate infection in a woodchuck hepatitis virus infectivity model.³⁴ It has also been shown that the X protein can function as a

Figure 3. The HBV life cycle. Entry of the HBV virion into the cell is a poorly defined process that is presumably receptor-mediated and leads to uncoating and transport of the capsid to the nucleus, where evidence suggests disassembly occurs, releasing the open circular viral genome into the nucleus where the second strand is completed and the ends of each strand are ligated. This leads to the production of a covalently closed circular DNA (cccDNA) molecule, which is the transcriptional template of the virus. Pol II-driven transcription results in production of the 4 viral RNAs that are thought to be actively transported out of the nucleus via shared sequences at the 3' end of the transcripts that apparently interact with cellular RNA export proteins. Once in the cytoplasm, the transcripts are translated into the corresponding proteins as shown. The precore protein contains a leader sequence that transports it into the endoplasmic reticulum (ER) where it is further processed and eventually secreted as HBeAg. The envelope proteins traverse the ER membrane as integral membrane proteins as shown. The core and polymerase proteins assemble around the pregenomic RNA (pRNA) to form HBV RNA-containing capsids within which the RNA is reverse transcribed to produce the first strand viral DNA that serves as the template for second strand DNA synthesis. While the RNA-containing capsid is maturing into a DNA-containing capsid it migrates bidirectionally within the cytoplasm. One pathway terminates at the ER membrane where it interacts with the envelope proteins which trigger an internal budding reaction resulting in the formation of virions that are transported out of the cell by the default secretory pathway. The second pathway transports the maturing capsid to the nucleus to amplify the pool of cccDNA.

cofactor for the development of hepatocellular carcinoma when it is overexpressed as a transgene in a mouse strain that has an unusually high baseline incidence of HCC.³⁵

defined the pathogenetic and antiviral functions of HBV-specific CTLs that we injected into HBV transgenic mice that express the corresponding viral proteins in their liver.

To characterize the human CTL response to HBV, we had to develop the technology to detect low abundance

The T Cell Response to HBV

As summarized in Figure 4, my laboratory has been interested in defining the host-virus interactions that determine the outcome of HBV infection, especially the role of the cytotoxic T lymphocyte (CTL) response, because antiviral T cells are believed to play a major role in control of HBV infection by virtue of their capacity to identify and kill virus-infected cells. We took two complementary approaches to this objective. First, to determine whether the CTL response plays a role in viral clearance and/or disease pathogenesis, we characterized and compared the quality and magnitude of the CTL response to HBV in patients who resolve the infection and patients who become persistently infected. Second, we established and

OVERVIEW

- Define the host-virus interactions that determine the outcome of HBV infection, especially the role of the CTL response.
- Characterize the CTL response in patients who clear HBV or become persistently infected.
- Define the pathogenetic and antiviral functions of HBV-specific CTLs following adoptive transfer into HBV transgenic mice.

Figure 4. Overview.

Figure 5. HBV-specific CTL response during acute and chronic infection. The CTL response to 5 HLA A2-restricted epitopes derived from the viral core, envelope, and polymerase proteins is indicated by vertical bars. Each set of bars represents the cytolytic activity of 8 replicate assays for each peptide in each patient. Acutely infected patients typically respond vigorously to multiple epitopes, as shown, and the response persists for many decades in patients who are convalescent from acute infection. In contrast, the CTL response is characteristically weak or undetectable in chronically infected patients. However, it is detectable in previously infected patients who clear the virus in response to interferon therapy, indicating CTLs are present in chronically infected patients but either too infrequent to be detected or functionally suppressed.

CTLs for this virus, which doesn't grow in tissue culture and therefore doesn't provide a ready supply of infected target cells. We succeeded by stimulating peripheral blood mononuclear cells (PBMCs) from infected patients with synthetic peptides derived from the various viral proteins and by testing the expanded T cells for the ability to kill autologous Epstein-Barr virus-transformed B cell lines that we infected with a panel of recombinant vaccinia viruses that express the corresponding viral protein. Using this technique, we showed (Figure 5) that all of the viral proteins are targeted by class I restricted CD8-positive CTL in patients with acute hepatitis who ultimately clear the virus, whereas the CTL response is relatively weak and more narrowly restricted in persistently infected patients.² The association between a strong, multifaceted T cell response with acute hepatitis and viral clearance suggests a causal relationship between these events. However, it does not prove causality, nor does it reveal the mechanisms responsible for viral clearance or disease pathogenesis during HBV infection.

This requires interventional studies that are not possible in humans but that can be pursued in appropriate animal models (see below).

Despite the vigor of the T cell response to HBV during acute viral hepatitis, we showed that very low levels of HBV DNA, detectable only by nested polymerase chain reaction, are present in the serum and PBMC of these patients for several decades after complete clinical and serological resolution of disease.³⁶ Interestingly, recent studies indicate that long term persistence of trace amounts of viral DNA is associated with equally long term persistence of HBV-specific CTL in these individuals,³⁷ suggesting that the viral DNA is transcriptionally active and able to produce viral antigens that actively maintain the CTL response, perhaps for life. This implies that traces of virus, not only viral DNA, may persist indefinitely in the face of the CTL response after recovery from acute viral hepatitis. It also implies that acute and chronic HBV infection may simply be points on a quantitative spectrum, rather than qualitatively different from each other.

The fascinating ability of HBV to persist at low levels after acute infection and to simultaneously maintain and evade the immune response is a matter of considerable interest, because it challenges the conventional wisdom that sterilizing immunity occurs after recovery from acute viral infections and because it raises the possibility that continuing antigenic stimulation may be needed to maintain immunological memory in a state of readiness for future viral exposure. This is a controversial area in viral immunology, although it has recently been confirmed in a number of other infections.^{38,39}

Viral Clearance by Destruction of Infected Cells

A large body of evidence suggests that the vigor of the CTL response to HBV is the principal determinant of viral clearance in infected patients.² It is widely believed that the CTL response clears viral infections by killing infected cells. Although this is probably true for many viruses, it may not be possible for the CTL response to eradicate infections in which the number of infected cells outnumber the antigen-specific T cells by several orders of magnitude. This appears to be the case for at least some of the hepatitis viruses, especially HBV, which can infect virtually all of the hepatocytes in the liver.

The reasons for this are as follows. For CTL to kill a target cell *in vivo*, the CTL must be induced by encountering antigen in an immunologically stimulatory microenvironment (eg, lymphoid tissue), enter the circulation and eventually the infected tissue, stop, migrate past any tissue barriers (eg, endothelial cells, basement membranes), reach the infected cells, recognize their cognate antigens, kill the infected cells one at a time, move on to find the next target cell, and kill it before the CTL are triggered to die themselves by the process of activation-induced cell death.⁴⁰ Thus, viral elimination by CTL-mediated killing is not nearly as efficient *in vivo* as one might assume from the ease with which CTL can kill target cells *in vitro*, where the only functions needed are antigen recognition and delivery of one or more death signals, and where the target cells are crowded around the CTL and have been highly selected for their exquisite sensitivity to those signals.

In addition, consider that there are approximately 10^{11} infectible hepatocytes in the human liver and that all of them can be infected by HBV.⁴¹ Consider, also, that there are approximately 10^{12} lymphocytes in the entire body and that the HBV-specific CTL precursor frequency at the height of the CTL response in a strongly reactive patient with acute hepatitis is rarely greater than 10^{-4} ; usually it is much lower.⁴² Hence, there should be no more than 10^8 HBV-specific CTL in the entire body at any point in time. Therefore, if every HBV-specific CTL in the entire body were to enter the liver at the same time (highly unlikely), and if most of the hepatocytes were infected (very common), there would be 1 specific CTL in the liver for every 1000 infected hepatocytes. Even considering the temporally extended dynamics of the CTL response, under these conditions it is difficult to imagine that viral

clearance can be achieved simply by the destruction of all of the infected cells. Even if only 10% of the hepatocytes were infected, the effector-to-target cell ratio in the liver would be 1:100, well beyond the cytolytic capacity of a single T cell. Even at the 1% infection level the ratio would be 1:10, which would require every CTL to find and kill 10 infected hepatocytes that are widely separated from each other by a 100-fold excess of uninfected cells to clear the infection. Although efficient cytolysis can sometimes be achieved *in vitro* at this effector-to-target cell ratio, it is very unusual; *in vitro*, the CTL are surrounded by target cells, all of which express their cognate antigen, so their task is much simpler than it is *in vivo*. Thus, as the number of infected cells decreases, the challenge of finding them increases, further contributing to the difficulty of clearing an infection by killing alone. Furthermore, if clearance were due entirely to the destruction of infected cells, one might reasonably expect the incidence of fatal acute hepatitis to be much higher than it is during HBV infection. For all of these reasons, we suggest that although the liver disease in viral hepatitis is certainly due to the destructive potential of the CTL response, viral clearance probably requires additional CTL functions besides their ability to kill infected cells.

CTL-Induced Liver Disease in HBV Transgenic Mice

Proof that an MHC-restricted cytolytic immune response to viral encoded antigens expressed at the surface of the hepatocyte plays an important role in viral clearance and, in the pathogenesis of HBV-induced liver disease, required the development of a readily manipulable small animal model in an immunologically well defined species. Because HBV does not infect such animals, we decided to produce HBV transgenic mice to achieve this objective.⁴³ Initially, we showed that mice that express HBV envelope proteins in their hepatocytes develop acute viral hepatitis after adoptive transfer of CD8-positive, MHC class I restricted, envelope (HBsAg)-specific CTL lines and clones.^{44,45} Furthermore, we showed that the disease progresses through an orderly series of clearly definable steps. The first step occurs within 1 hour of CTL administration, with antigen recognition by the CTL and delivery of a signal that results in the death of CTL-associated hepatocytes by apoptosis (Figure 6a), causing the formation of acidophilic Councilman bodies (apoptotic hepatocytes) that are characteristic of acute viral hepatitis in man.⁴⁶ The second step begins between 4 and 12 hours after CTL injection, when many host-derived inflammatory cells are recruited into the vicinity of the CTLs (Figure 6b), resulting in the formation of necro-inflammatory foci and the destruction of additional hepatocytes. Importantly, the effector-to-target cell ratio *in vivo* in these experiments is very low ($\sim 1/30$ – $1/100$), so only a small fraction of the hepatocytes are killed by the combined effects of the CTLs plus the ensuing inflammatory response.^{47–49} However, if many HBsAg-positive ground glass hepatocytes are present in the liver, a third process ensues in which the animal may die from fulminant hep-

Figure 6. CTL-induced apoptosis and inflammation in HBV transgenic mice. **A:** Within 1 hour after intravenous injection of murine HBsAg-specific CTLs into HBV transgenic mice that express all viral gene products, replicate the viral genome and produce infectious virions, the CTLs recognize processed antigenic peptides presented by class I molecules on the surface of hepatocytes and stimulate them to undergo apoptosis. In this experiment, bromodeoxyuridine (BrdU)-labeled CTLs (**arrow**) were injected and the liver was stained with an anti-BrdU specific antibody imparting a brown stain to the CTL. Note the condensation and fragmentation of the cytoplasm and nucleus of the hepatocyte (**asterisk**), indicating apoptosis. **B:** Between 24 and 48 hours later, the CTL-induced necroinflammatory disease is maximal and the CTLs (**arrow**) have recruited a mixed population of host-derived, HBV-nonspecific inflammatory cells (**arrowheads**), many of which are associated with necrotic and apoptotic hepatocytes at a distance from the CTL. Under the conditions of this experiment, the CTLs and associated foci are widely scattered such that fewer than 10% of the hepatocytes are killed. Note that the hepatocytes surrounding the inflammatory focus are histologically normal.

atitis because ground glass cells are exquisitely sensitive to destruction by IFN- γ , and this cytokine is actively secreted by CTLs after antigen recognition.³² The striking similarities between the immunopathological and histopathological features of this model and acute viral hepatitis in man suggest that similar events may contribute to the pathogenesis of the human disease as well.

CTL-Induced Viral Clearance in HBV Transgenic Mice

Interestingly, the CTLs do not induce a second episode of hepatitis if they are readministered to the mice less than 4 weeks after the first CTL injection.⁵⁰ While studying the basis for this observation we learned that, in addition to killing some of the hepatocytes, the CTL also down-regulated the expression and replication of HBV by all of the hepatocytes in the liver without killing them.⁴⁹ In subsequent experiments using transgenic mice that contain the complete viral genome as recipients of CTLs,⁴⁷ we

demonstrated that all of the viral RNAs, their translation products (HBcAg, HBeAg, and HBsAg), nucleocapsids, and episomal replicative DNA intermediates, are susceptible to this remarkable antiviral effect (Figure 7). Two lines of evidence suggest that this antiviral process is noncytopathic and that it is mediated by inflammatory cytokines. First, it can be blocked by the prior administration of antibodies to IFN- γ or tumor necrosis factor α (TNF- α) without reducing the severity of the liver disease. Second, it can be reproduced by perforin-deficient CTLs and Fas ligand-deficient CTLs that do not cause hepatitis in these animals, but it cannot be reproduced by IFN- γ -deficient CTLs, even though they cause hepatitis (Figure 8).⁴⁷ In recent studies (McClary H, Koch R, Chisari FV, Guidotti LG: *J Virol*, in press) we reproduced the antiviral effect by injecting wild-type CTLs into HBV transgenic mice whose IFN- γ and TNF- α receptor (p55) had been knocked out, indicating that it is the IFN- γ the CTLs produce themselves that inhibits HBV replication, not the IFN- γ produced by the inflammatory cells that the CTLs

Figure 7. Noncytotoxic antiviral effect of the CTLs. **A** displays the presence of HBcAg (red stain) in the nucleus and the cytoplasm of hepatocytes in an HBV transgenic mouse before the injection of HBV-specific CTLs. **B** displays the absence of HBcAg from the liver of an HBV transgenic mouse 5 days after the injection of HBV-specific CTLs. The **upper** and **lower insets** represent Northern and Southern blots, respectively, of liver RNA and DNA from the same control (**left side**) and CTL-injected (**right side**) mouse livers stained in **A** and **B**. GAPDH RNA and integrated transgene DNA serve as loading controls for the Northern and Southern blots, respectively. Note that virtually 100% of the viral RNA, replicative DNA intermediates, and core protein disappear from the liver after CTL injection. Under the conditions of this experiment, less than 10% of the hepatocytes were destroyed. Thus, viral clearance from the rest of the hepatocytes was not due to their destruction.

recruit. In other experiments we showed that the regulatory effect of the CTLs becomes independent of IFN- γ during the first 24 hours after CTL administration, suggesting that the cytokines activate a regulatory cascade that ultimately delivers the final inhibitory signal to the hepatocyte. Furthermore, using HBV transgenic mice whose inducible nitric oxide synthase (iNOS) allele has been knocked out (Guidotti L, McClary H, Moorhead J, Chisari FV; *J Exp Med*, in press), we showed that nitric oxide inhibits HBV replication and that it is a downstream mediator of the antiviral effect of IFN- γ .

At the effector level, therefore it would appear that the CTL response can activate two different pathways to eliminate a virus, either by killing the infected cells or by eliminating the virus from within the cell without killing it. These alternate scenarios can be activated simultaneously as a consequence of antigen recognition, as illustrated in Figure 9. According to this scenario, viral clearance depends on the development of a vigorous intrahepatic immune response, with the severity of the associated liver disease being determined by the num-

ber of infected hepatocytes and the balance between the cytopathic and antiviral regulatory effects of the intrahepatic inflammatory cells. If the T cell response is strong and the number of infected cells is low, viral clearance should occur rapidly and efficiently, with little evidence of liver disease, simply by killing the infected cells. Even a strong T cell response may not be able to clear a massive viral infection, however, unless the curative limb of the response is called into play. In the absence of this component, the cytopathic function of the immune response may not be able to eliminate all of the infected cells, leading to persistent infection and chronic liver disease. On the other hand, if the T cell response is quantitatively suboptimal, the virus could persist even if the appropriate antiviral cytokines are produced, since, at insufficient levels, they will suppress viral gene expression without eliminating it, thereby making the virus less visible to the immune system.

We are currently attempting to determine and characterize the steps in the viral life cycle (Figure 3) that are interrupted by the cytokines, and to identify the cytokine-

Figure 8. IFN- γ mediates the antiviral effect of the CTLs. Mice were injected with equal numbers of HBV-specific CTL clones that recognize the same viral epitope with equal affinity. CTL clones were produced in wild-type mice and in mice that lack the genes for either Fas ligand, perforin, or IFN- γ . As shown in **A**, the wild-type (wt) and IFN- γ -deficient (IFN- γ ⁻) CTL clones cause a transient episode of hepatitis, indicated by elevated serum alanine aminotransferase (ALT) activity. In contrast, the Fas ligand-deficient (FasL⁻) and perforin-deficient (Perf.⁻) CTL clones failed to cause hepatitis. As shown in **B**, the wt clone abolished HBV replicative intermediates from the liver while leaving the integrated transgene unaffected, since most cells were not destroyed. Similarly, the Perf.⁻ and FasL⁻ CTL clones also inhibited HBV replication, even though they didn't kill any hepatocytes, as shown in **A**. Importantly, the IFN γ ⁻ clone did not inhibit HBV replication, even though it caused hepatitis. Similar results were obtained in separate experiments in which the antiviral effects of the wt CTLs were completely blocked by a cocktail of antibodies to IFN- γ and TNF- α , which had no effect on the cytopathic effect of the CTLs *in vivo*. These results demonstrate that the genetic and functional levels that the cytopathic and antiviral functions of the CTLs are independent of one another, although both functions require antigen recognition by the CTLs.

regulated hepatocellular genes that inhibit HBV gene expression and replication in this system. At the moment, we know that the cytokines inhibit viral gene expression by a posttranscriptional mechanism that destabilizes the viral mRNA in the nucleus of the cell,⁵¹ and we have shown that this is associated with the cytokine-induced proteolytic cleavage of a cellular HBV RNA-binding protein that stabilizes the viral RNA under baseline conditions.^{52,53} We also know that the cytokines inhibit viral replication by posttranslationally eliminating viral nucleocapsid particles, within which replication occurs, from the cytoplasm of the hepatocyte (Wieland S, Guidotti LG, Chisari FV, *J Virol*, in press). We do not know, however, whether the cytokines prevent nucleocapsid assembly or accelerate nucleocapsid degradation, and studies are underway to determine which of these mechanisms is operative.

It is important to note that other intrahepatic stimuli, in addition to CTLs, can initiate these antiviral events, as long as they trigger the production of antiviral cytokines in the liver. For example, we showed that HBsAg-specific class II restricted T cell clones can trigger the same effects by secreting IFN- γ in the liver when they recognize antigen presented by hepatic macrophages.⁵⁴ We also showed that systemically administered recombinant interleukin-12 (IL-12) can inhibit HBV replication by inducing the production of IFN- γ in the liver.⁵⁵ Since IFN- γ is a powerful macrophage activator, it is possible that its antiviral effect is mediated by TNF- α produced by acti-

vated macrophages. In keeping with this notion, we have shown that recombinant TNF- α also inhibits HBV gene expression in these mice,⁵⁶ as does interleukin-2 (IL-2), the regulatory effects of which are mediated by TNF- α ⁵⁷ by posttranscriptionally accelerating the degradation of HBV mRNA⁵⁸ as described above. One might predict from the foregoing that coinfection or superinfection of the HBV-infected liver by other pathogens could facilitate the clearance of HBV if the other pathogens induce the local production of the antiviral cytokines to which HBV is susceptible. Precisely these events have been shown to occur in the HBV transgenic mice during lymphocytic choriomeningitis virus,⁴⁸ adenovirus, and cytomegalovirus⁵⁹ infection of the liver, reminiscent of isolated case reports suggesting that superinfection by hepatitis A virus or hepatitis C virus (HCV) is sometimes associated with clearance of HBV in chronically infected patients.^{60,61} The potential of these antiviral pathways to be exploited for therapeutic purposes is self-evident.

These results suggest that a strong intrahepatic immune response to HBV during a natural viral infection can suppress viral gene expression and replication and, if the supercoiled viral genome is also eliminated by this process, perhaps even cure infected hepatocytes of the virus without killing them. Importantly, this illustrates that the infected cells can become active participants in the antiviral response by responding to cytokine-induced signals and activating specific intracellular pathways that interrupt the viral life cycle. This is a significant departure

Intracellular Inactivation of HBV

Figure 9. Noncytolytic clearance of HBV from the hepatocyte by T cell-derived cytokines. On antigen recognition, CD8-positive CTL deliver an apoptotic signal to their target cells, killing them. They also secrete IFN- γ and TNF- α , cytokines that abolish HBV gene expression and viral replication *in vivo*, curing them. The curative effect of the CTL response is more efficient than its destructive effect. The outcome of an infection may depend on the relative balance of these two effects, with a predominantly curative response leading to viral clearance and a predominantly destructive response leading to viral persistence and chronic liver disease. Importantly, if the curative process only partially inhibits viral gene expression and replication, it could paradoxically lead to viral persistence by reducing the immunological visibility of the virus without removing it. Note that antiviral cytokines produced by non-T cells during acute infection or by HBV-nonspecific T cells in the event of a superinfection could also inhibit HBV replication by a bystander mechanism. This is common during hepatitis delta virus superinfection of patients chronically infected by HBV, and it has been described during hepatitis A virus and HCV superinfection as well.

from current dogma, which views the infected cell merely as a victim of the viral infection. As a corollary of this hypothesis, the data suggest that a weak immune response or incomplete viral inactivation could contribute to viral persistence and chronic liver disease by reducing the expression of viral antigens sufficiently for the infected cells to escape immune recognition.

Noncytopathic Clearance of HBV during Acute Infection in Chimpanzees

The transgenic mouse studies suffer from two important limitations. First, the mice are not infected by HBV, so the observations are limited to biochemical aspects of viral

replication and gene expression, and they fail to examine the effects of the cytokines in the context of viral entry and spread. Second, for unknown reasons, the mice do not produce the episomal covalently closed circular HBV DNA (cccDNA) species that serves as the viral transcriptional and that must be eliminated for viral clearance to occur. Accordingly, confirmation of the hypothesis that viral clearance during HBV infection reflects noncytopathic processes initiated by the immune response requires analysis of these events in the liver of HBV-infected animals that produce cccDNA. Chimpanzees are ideal for these purposes because they are infectible by HBV¹ and they are known to mount a cellular immune response to HBV similar to that observed in acutely infected humans.⁶² Therefore, we infected two healthy young adult HBV-seronegative chimpanzees with HBV and compared the kinetics of viral clearance and the kinetics of liver disease by obtaining liver biopsies every week for 24 weeks after inoculation. Both chimpanzees developed typical cases of acute, self-limited viral hepatitis. Importantly, the viral DNA (including the cccDNA) disappeared from the serum and the liver from both animals several weeks before the peak of the disease (Figure 10). Interestingly, the disappearance of viral DNA from the liver coincided with the induction of IFN- γ , which preceded the major influx of T cells. These results demonstrate that the destruction of the hepatocytes could not be responsible for the reduction of viral DNA. They also suggest that the early noncytopathic control of HBV replication in these animals, which was associated with the induction of IFN, may be due to the influx into the liver of IFN- γ -producing non-T cells, perhaps natural killer cells, that can recognize infected cells in the absence of MHC class I expression. In this scenario, the IFN- γ produced by the influx of natural killer cells could perform dual functions by inhibiting HBV replication, thereby reducing the number of infected hepatocytes, and by up-regulating MHC class I, thereby permitting the remaining infected cells to present viral antigens to the antigen-specific CTLs that complete the process by killing the residual targets and causing the disease recognized as viral hepatitis. This tissue-sparing, noncytolytic antiviral process can be viewed as a host survival strategy to control infections of vital organs that would otherwise be destroyed if the only way to eliminate the infections were to kill all of the infected cells. Interestingly, by down-regulating viral antigen expression, the same process could also function as a viral evasion strategy and contribute to viral persistence. Indeed, both scenarios might be correct, and they could even be operative at the same time in the same individual in view of the recent discovery that traces of HBV can persist for several decades after complete serological and clinical recovery from acute viral hepatitis.¹⁹ If so, this noncytolytic process may be strongly favored during evolution and possibly extend to other pathogens, since it provides a strong survival advantage for both virus and host.

With this in mind, we performed a study to determine whether cytokines are involved in clearance of lymphocytic choriomeningitis virus (LCMV) from the liver and other tissues.⁶³ The results showed that LCMV is cleared

Figure 10. Noncytolytic clearance of HBV in an acutely infected chimpanzee. In this experiment, serum and needle liver biopsies were obtained on a weekly basis after inoculation of a chimpanzee with HBV-positive plasma from an HBV transgenic mouse. The chimpanzee became transiently infected as indicated by the appearance and eventual clearance of HBV DNA from the liver. The chimpanzee also developed an episode of acute hepatitis as seen by the transient elevation of serum ALT activity. Note that the kinetics of viral clearance (determined by competitive polymerase chain reaction in the **top panel** and by Southern blot in the **middle panel**) preceded the kinetics of disease activity by several weeks, indicating that the two events are independent during acute HBV infection in this model, similar to the observations in the HBV transgenic mice. Note also, that the cccDNA species as well as the viral replicative intermediates disappeared from the liver with similar kinetics, suggesting that the cccDNA is susceptible to noncytolytic clearance mechanisms as well as the replicative intermediates. Finally, note that the decrease in viral DNA coincides with the appearance of IFN- γ mRNA in the liver, whereas the liver disease correlates primarily with the appearance of CD3 mRNA, a T cell marker. This suggests that antiviral inflammatory cytokines produced by non-T cells may play an important role in the early viral clearance process, while the disease is more closely related to the influx of T cells into the liver.

from the hepatocyte noncytopathically when IFN- γ is induced in the liver by antigen-nonspecific stimuli. These results indicate that, like HBV, LCMV is also susceptible to intracellular inactivation by cytokine-induced antiviral mechanisms that are operative in the hepatocyte. However, LCMV is not cleared from intrahepatic nonparenchymal cells or splenocytes, indicating that, unlike the hepatocyte, these cells do not produce the factors required to inactivate LCMV. Antiviral mechanisms like these may have evolved to maintain the functional integrity of vital organs, such as the liver, in the face of massive infection.

Mechanisms of HBV Persistence

For a noncytopathic virus to persist, it must either overwhelm (or not induce) an effective antiviral immune response, or it must be able to evade it (Figure 11). All of these scenarios could be operative in patients chronically infected by HBV. Indeed, neonatal tolerance is probably responsible for both the lack of an antiviral immune response and viral persistence after mother-infant transmission, which is the most common antecedent of per-

sistent HBV infection worldwide.² The immunological basis for viral persistence during adult onset infection is not well understood. Perhaps the simplest explanation is quantitative, based on the kinetics of infection relative to the induction of a CTL response during the early days of an infection. For example, viral persistence would be predicted if the size of the inoculum or the replication rate of an incoming virus exceeds the kinetics of the immune response, such that the effector-to-target cell ratio favors the virus even when the CTL response is fully in place. However, since the CTL response is much less vigorous in chronically infected patients than it is during acute infection,⁶⁴⁻⁶⁷ other factors must be involved as well. Reasonable candidates are the induction of peripheral tolerance or exhaustion of the T cell response by the high viral load that characterizes most persistently infected patients. Additionally, virus-specific CTLs, which might otherwise become activated by antigen recognition in the immunostimulatory context of secondary lymphoid organs, might be inactivated if antigen is presented in the absence of costimulatory signals in the liver.

Other candidate mechanisms that could contribute to viral persistence include infection of immunologically privileged sites, viral inhibition of antigen presentation, selective immune suppression, down-regulation of viral gene expression, and viral mutations that abrogate, anergize, or antagonize antigen recognition by virus-specific T cells.² There is some evidence that privileged sites may play a role, because HBV does infect extrahepatic tissues. Also, we have shown that circulating HBV-specific CTL can cause hepatitis but not nephritis in HBV transgenic mice that express the virus in the liver and the kidney, due to the limited access of the CTLs to antigen-positive cells present on the other side of microvascular barriers that exist in the kidney but are not present in the liver sinusoid.⁴⁴ Additionally, it has been suggested that infected cells that express Fas ligand can protect themselves against CTL-mediated injury by actively destroying the CTLs via the same Fas ligand-Fas receptor pathway that CTLs use to kill their target cells, but in reverse.⁶⁸ Importantly, it appears that hepatocytes can be induced to express Fas ligand during an inflammatory response.⁶⁹ If so, individuals whose hepatocytes express Fas ligand most efficiently would be most likely to delete their HBV-specific CTL and, therefore, become chronically infected. An interesting correlate of this scenario is the theoretical possibility that HBV itself might be able to induce Fas ligand expression by the hepatocyte, thereby deleting the HBV-specific CTL population when it enters the liver. In either case, Fas ligand induction would have to occur without inducing hepatocyte fratricide by virtue of Fas ligand-Fas interactions between adjacent cells.⁶⁹ Alternatively, HBV could theoretically down-regulate Fas expression, rendering the infected hepatocyte relatively resistant to destruction by the CTL. All of these theories are testable, but they are strictly speculative at present.

Certain viruses (eg, poxviruses, adenoviruses, herpesviruses) have evolved the ability to inhibit antigen presentation or to suppress or neutralize antiviral cytokines as survival strategies.^{70,71} Thus far, however, there is no evidence that these processes are operative during HBV

Figure 11. Mechanisms of viral persistence.

infection. As discussed earlier, however, inflammatory cytokines, especially IFN- γ , suppress HBV gene expression and replication, which could contribute to viral persistence if the effect is incomplete or if the virus infects an whose immune response to HBV does not produce this cytokine. Indeed, the cytopathic potential of the CTL in these individuals would trigger hepatitis, whereas the failure of their CTL to produce the appropriate cytokines might contribute to viral persistence. Analysis of cytokine expression in the liver of patients with chronic HBV infection should clarify this interesting hypothesis.

The role of viral escape mutations as a cause of viral persistence has attracted considerable interest in recent years. Many conditions must be fulfilled, however, for a mutant virus to be selected by CTL-mediated immune pressure.² Perhaps the most important condition is the occurrence of a strong CTL response that is focused on a single viral epitope. This scenario would favor the outgrowth of variant viruses because they would not otherwise be visible to the immune system. This type of CTL response is unusual, however, during HBV infection, when the CTL response is typically vigorous and multispecific during acute hepatitis and weak or undetectable during chronic hepatitis.^{42,65,66,72} Accordingly, mutational inactivation of CTL epitopes is extremely uncommon during chronic HBV infection.⁷³

Nonetheless, strong, narrowly focused CTL responses do occur occasionally in these patients,²⁰ and in this setting viral escape mutations can occur.⁷⁴ Vigorous oligoclonal expansions of T cells have been described in other persistent viral infections, especially HIV⁷⁵ and Epstein-Barr virus.⁷⁶ Even in these infections, however, viral mutations that affect recognition of an epitope by some CTL clones do not automatically affect all CTL clones specific for the same epitope, because different T cell clones can bind different residues in the same epitope.^{77,78} Although CTL escape can confer a strong survival advantage, it is important to emphasize that selection of escape variants in all of these infections occurs in the setting of a pre-existing persistent infection; ie, viral persistence probably leads to selection of escape variants, not the reverse.

The situation may be somewhat different in early chronic HIV infection where the CTL response is characteristically very strong, yet is unable to clear the virus.^{75,76}

The incredibly high rate of HIV production and the exceptionally high mutation rate of this virus may cause so many different viruses to be generated each day that they exceed the capacity of the immune system to respond effectively simply on a numerical basis.⁷⁸ In this regard, the ostensibly vigorous immune response HIV appears to be unable to compete with the capacity of the virus to generate mutants. Mutational inactivation of CTL epitopes might thus play an earlier and more important role in the establishment of viral persistence for HIV than for HBV.⁷⁹ It is important to emphasize, however, that the overwhelming rates of viral replication and spread relative to the ability of the immune system to produce enough CTLs to reach and destroy all of the infected cells, plus the immunosuppressive effects of the virus itself, are more important than viral mutation for the development of persistent infection.

The situation may be different again during chronic HCV infection where an extensive quasispecies of viral variants can coexist with a multispecific CTL response^{80–83} that is intermediate in strength between the response of patients chronically infected by HBV and those infected by HIV. Unlike HBV and HIV, where the viral load is high, the viral titer is very low during chronic HCV infection,⁸⁴ so viral persistence cannot easily be blamed on an overwhelming infection in this instance. Therefore, escape mutants may play a greater role in the primary establishment of HCV persistence than is likely for HBV. Importantly, CTL escape has been observed in a chronically HCV-infected chimpanzee.⁸⁵ However, the extent to which the mutation contributed to or was a consequence of persistent HCV infection in this case remains to be determined.

In view of the multispecificity of the CTL response to most persistent viruses, current data favor the notion that negative selection of CTL escape mutants is most likely to occur after a persistent infection is already established. In this setting, viral mutations could solidify the chronic nature of the infection and perhaps even make it irreversible. Whether such mutations can also serve as the primary cause of viral persistence in the context of a multispecific T cell response remains to be proven.

Immune Pathogenesis of Hepatocellular Carcinoma

The mechanisms responsible for malignant transformation in chronic HBV infection are not well defined, and both viral and host factors have been implicated in the process. On the one hand, all cases of HCC occur after many years of chronic hepatitis which could, theoretically, provide the mitogenic and mutagenic environment to precipitate random genetic and chromosomal damage and lead to the development of HCC. On the other hand, most tumors contain clonally integrated HBV DNA and microdeletions in the flanking cellular DNA which could, theoretically, deregulate cellular growth control mechanisms.⁸⁶ Furthermore, the HBV X gene product has been shown to transactivate cellular genes associated with cellular growth control^{87–89} and inhibit p53 gene function

*in vitro*⁹⁰, suggesting that deregulated X gene expression from integrated fragments of subviral DNA could play a role in hepatocarcinogenesis.⁹¹ Similarly, C-terminally truncated viral envelope proteins expressed from integrated subviral DNA may have transactivating activity^{92,93} and could, potentially, contribute to carcinogenesis in chronic HBV infection. Like retroviruses, however, HBV integration does not occur in resting hepatocytes; so if HBV integration plays a role in hepatocarcinogenesis, antecedent events must occur that trigger hepatocellular turnover.

In an effort to clarify the carcinogenic potential of chronic hepatitis, we previously showed that transgenic mice that produce hepatotoxic quantities of the HBV large envelope polypeptide^{43,94-96} display hepatocellular injury, regenerative hyperplasia, chronic inflammation, Kupffer cell hyperplasia, oxygen radical production, glutathione depletion, oxidative DNA damage, transcriptional deregulation and aneuploidy that inexorably progresses to HCC.⁹⁵⁻¹⁰⁰ While those studies demonstrated that HBV can cause hepatocellular carcinoma in the absence of insertional mutagenesis, X gene expression or genotoxic chemicals, they did not prove that chronic immune-mediated hepatitis was a procarcinogenic stimulus in itself. To determine whether hepatocellular carcinoma can be triggered by a chronic, virus-specific immune response, we developed a model of chronic immune-mediated liver disease using transgenic mice that express nontoxic concentrations of the HBV envelope proteins in the hepatocyte. Similar to human chronic HBsAg carriers, these mice are immunologically tolerant to HBsAg and they develop no evidence of liver disease except ground glass hepatocytes during their lifetime. The mice were thymectomized, lethally irradiated and reconstituted with bone marrow and spleen cells either from syngeneic nontransgenic donors that were previously immunized with a recombinant vaccinia virus that expresses HBsAg and displayed HBsAg-specific CTLs and anti-HBs antibodies, or from immunologically tolerant transgenic donors. All results were compared with unmanipulated, age- and sex-matched transgenic mice. Only mice that were reconstituted with immunologically primed nontransgenic immune systems developed acute hepatitis and cleared HBsAg from their serum. Subsequently, all of these mice developed chronic hepatitis and HCC.¹⁰¹

The pathogenetic importance of immune-mediated hepatocellular injury in hepatocarcinogenesis in this study is strengthened by the fact that hepatocellular carcinoma occurs in the context of necrosis, inflammation and regeneration (cirrhosis) in several human liver diseases other than hepatitis B, including chronic hepatitis C,¹⁰² alcoholism,¹⁰³ hemochromatosis,¹⁰⁴ glycogen storage disease,¹⁰⁵ α -1-antitrypsin deficiency,^{106,107} and primary biliary cirrhosis.¹⁰⁸ Irrespective of etiology or pathogenesis, therefore, it would appear that chronic liver cell injury is a premalignant condition that initiates a cascade of events characterized by increased rates of cellular DNA synthesis and production of endogenous mutagens coupled with compromised cellular detoxification and repair functions. If these processes are sustained for a

Hypothesis Hepatocarcinogenesis in HBV Infection

Figure 12. The chronic injury → HCC hypothesis. According to this hypothesis, a vigorous (+++) immune response to HBV leads to viral clearance while an absent (–) immune response leads to the “healthy” carrier state, and an intermediate (+) immune response produces chronic hepatitis. This indolent necroinflammatory liver disease is characterized by chronic liver cell necrosis which stimulates a sustained regenerative response. The inflammatory component includes activated macrophages that are a rich source of free radicals. The collaboration of these mitogenic and mutagenic stimuli has the potential to cause cellular and viral DNA damage, chromosomal abnormalities, genetic mutations, etc., that deregulate cellular growth control in a multistep process that eventually leads to hepatocellular carcinoma.

sufficiently long period of time, they would be expected to cause the multiple genetic and chromosomal changes necessary to trigger the development of hepatocellular carcinoma (Figure 12).

While these associations strongly suggest that chronic necroinflammation may be procarcinogenic in regenerative tissues, they do not constitute proof of this concept. The experimental results, however, provide definitive evidence that HBV-specific chronic immune-mediated liver cell injury is sufficient to initiate and sustain the process of hepatocarcinogenesis in this model. Furthermore, they demonstrate that the immune response is procarcinogenic despite the absence of cofactors such as random, widespread viral integration, X gene expression or genotoxic agents that have been proposed to contribute to the development of HCC in man. Since the immunological, virological and histological features of this model closely resemble human chronic hepatitis, the results suggest that an ineffective immune response is the principal oncogenic factor during chronic HBV infection in man. It is ironic that the same T cell response that can eradicate HBV from the liver when it is strong can be procarcinogenic by triggering a chronic necroinflammatory liver disease when it is unable to completely terminate the infection. If this is correct, therapeutic enhancement of the T cell response to HBV in chronically infected patients should prevent HCC.

Summary and Conclusions

The diversity of clinical syndromes and disease manifestations associated with HBV infection strongly suggests

that the clinical outcome of this infection is determined by the quality and vigor of the antiviral immune response produced by the infected host. Antibodies to antigens expressed at the surface of virus particles can provide protection from initial infection and can prevent viral spread from cell to cell once infection is established. Antibody-mediated immune complex formation can contribute to extrahepatic syndromes in these patients and may even play a role in liver disease if they can bind to the surface of antigen-positive hepatocytes and recruit Fc receptor-positive killer cells, thereby mediating antibody dependent cellular cytotoxicity. CD4-positive helper T cells serve a critically important regulatory function by secreting a variety of cytokines that can facilitate B cell maturation, expansion, and antibody secretion or that foster the development of a strong CTL response. CD8-positive CTLs can kill infected cells by direct contact, triggering them to undergo apoptosis and recruiting antigen-nonspecific inflammatory cells that amplify their cytopathic potential. They also secrete cytokines when they recognize antigen in the infected tissue, some of which have the potential to inhibit the expression and replication of HBV in the hepatocyte. All limbs of the immune response must cooperate productively to terminate a viral infection. Individual differences in the efficiency of viral antigen processing by hepatocytes and professional antigen-presenting cells, or at the level of antigen recognition and responsiveness by B and T lymphocytes, will affect the strength of the antiviral immune response and the extent to which it contributes to viral clearance and liver disease.

Finally, chronic hepatitis appears to be due to a sub-optimal cellular immune response that destroys some of the infected hepatocytes and does not purge the virus from the remaining infected hepatocytes, thereby permitting the persisting virus to trigger a chronic indolent necroinflammatory liver disease that sets the stage for development of HCC.

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