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Intracellular Inactivation of the Hepatitis B Virus by Cytotoxic T Lymphocytes

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Summary

It is widely believed that viral clearance is mediated principally by the destruction of infected cells by CTLs. In this report, we use a transgenic mouse model of HBV replication to demonstrate that this assumption may not be true for all viruses. We find that adoptively transferred virus-specific CTLs can abolish HBV gene expression and replication in the liver without killing the hepatocytes. This antiviral function is mediated by IFNγ and TNFα secreted by the CTL or by the antigen-nonspecific macrophages and T cells that they activate following antigen recognition. These cytokines activate two independent virocidal pathways: the first pathway eliminates HBV nucleocapsid particles and their cargo of replicating viral genomes, while the second pathway destabilizes the viral RNA. Intracellular viral inactivation mechanisms such as these could greatly amplify the protective effects of the immune response, while failure of such mechanisms could lead to viral persistence or to the death of the host.

Introduction

The clearance of intracellular pathogens, especially noncytopathic viruses, by the immune response is widely thought to be mediated primarily by the destruction of infected cells by major histocompatibility complex (MHC) class I–restricted $CD8^+$ cytotoxic T lymphocytes (CTLs). However, upon antigen recognition such CTL can also secrete potent antiviral cytokines such as interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα), which can potentially cure viral infections without killing the infected cells. The importance of noncytodestructive curative functions of CTL in viral clearance is not known, but has been implied by indirect evidence in the past (reviewed by Lehmann-Grube et al., 1988; Martz and Howell, 1989). The relative importance of cytodestructive versus curative CTL functions is obscure even for lymphocytic choriomeningitis virus (LCMV), the prototypic noncytopathic virus that requires CTL for clearance (Byrne and Oldstone, 1984). For example, perforin-deficient mice are unable to clear certain LCMV strains (Kägi et al., 1994; Walsh et al., 1994), thus suggesting that the specific destruction of infected cells by CTL is required for viral clearance. However, the same strains of LCMV replicate uncontrollably in mice that lack IFNγ receptors (Müller et al., 1994) or that have been treated with IFNγ-specific antibodies (Moskophidis et al., 1994), suggesting that clearance of LCMV also requires the production of this cytokine and perhaps others. Furthermore, LCMV infection in mice can be cleared, without evidence of cytopathology (Gegin and Lehmann-Grube, 1992), before the onset of detectable cytolytic activity and by extremely small numbers of adoptively transferred CTL (Lehmann-Grube et al., 1985).

In hepatitis B virus (HBV)-infected humans, viral clearance is associated with the development of a vigorous cellular immune response and an acute necroinflammatory liver disease, termed viral hepatitis (reviewed by Chisari and Ferrari, 1995). Hence, it is widely assumed that HBV clearance is mediated principally by the destruction of infected cells by virus-specific MHC class I–restricted CTL. This assumption may be incorrect, however, for at least two reasons. First, the CTL response may not be able to reach, recognize, and destroy the vast number of infected hepatocytes (up to 10^{11} cells) in the liver by direct physical contact. Second, HBV clearance is usually associated with clinical recovery, not death, despite the fact that the virus can infect virtually all hepatocytes. Rapid and complete viral clearance, without massive hepatocellular necrosis, is also the rule following woodchuck hepatitis virus (WHV) infection, during which virtually all the woodchuck hepatocytes are infected (Kajino et al., 1994). For these reasons, we favor the notion that viral clearance during HBV infection may be mediated by noncytolytic antiviral effects of CTL, and that the associated liver disease is an unfortunate complication of CTL activation rather than a determining factor in viral clearance.

The experimental approaches to HBV pathogenesis have been hampered because the host range of HBV is limited to man and chimpanzees and because culture systems for the propagation of HBV do not exist. Recently, however, we produced transgenic mice that replicate the HBV genome and sustain the HBV lifecycle in their hepatocytes (Guidotti et al., 1995). We now report that HBV-specific $CD8^+$ CTL can abolish HBV gene expression and replication in the liver of these transgenic mice while killing only a small fraction of the hepatocytes. This effect is mediated by inflammatory cytokines such as IFNγ and TNFα, apparently activating the hepatocytes to eliminate cytoplasmic HBV nucleocapsid particles with their replicating viral genomes and to destabilize the viral RNA, thereby interrupting viral replication. All of these events occur in perfectly viable hepatocytes that are entirely normal cytologically.

Results

Adoptively Transferred CTLs Kill a Small Fraction of HBV-Positive Hepatocytes in the Transgenic Mouse Liver

Age- and serum HBsAg-matched transgenic males from lineages 1.3.32 and 1.3.46 (H-2^d) were injected intravenously with various numbers of several independently derived $CD8^+$ L^d-restricted HBsAg-specific CTL (Ando et al., 1993; Guidotti et al., 1994a). These mice express all of the HBV antigens, including HBsAg, in the vast majority of their hepatocytes (Guidotti et al., 1995). As exemplified in Figure 1A for CTL clone 6C2 and lineage 1.3.32, all CTL clones produced a mild, transient, cell dose–dependent necroinflammatory liver disease in both lineages, monitored biochemically as serum alanine aminotransferase (sALT) activity, a hepatocellular enzyme that is released into the circulation by necrotic hepatocytes. sALT levels began to rise within a few hours of CTL administration, reached maximum severity by day 3, and returned to baseline within 1 week. As expected, disease severity was CTL dose dependent; yet sALT activity was only modestly elevated in mice receiving the highest number (1×10^7) of cloned CTL (Figure 1), compared with previously reported mouse models of liver disease (Ando et al., 1993).

To estimate the fraction of total hepatocytes that are destroyed by the CTL during this disease, we compared the ALT profile of the CTL-injected mice with the ALT profile in normal mice that were injected with a lysate prepared from a known amount of liver. In this manner, we estimated that no more than 6%, 10%, or 24% of the hepatocytes were killed following the injection of 2.5×10^6, 5×10^6, and 1×10^7 CTL, respectively (data not shown).

Histological analysis of the livers at the peak of sALT activity (day 3) demonstrated necroinflammatory foci containing dead and degenerating hepatocytes, distributed principally in the periphery of the hepatic lobule, adjacent to the portal veins from which cells enter the parenchyma of the liver (see below). However, the vast majority of the hepatocytes, especially the centrilobular hepatocytes that replicate high level HBV in these animals (Guidotti et al., 1995), appeared histologically normal. As a specificity control, 1×10^7 HBsAg-specific CTLs were injected into nontransgenic littermates and into transgenic mice from lineage 1.3.32 that lack the H-2^d restriction element utilized by the CTL. No biochemical or histopathological evidence of liver disease was observed in any of these animals (data not shown).

Adoptively Transferred CTLs Clear All Traces of HBV Gene Expression and Replication from the Liver and Virions from the Serum of HBV Transgenic Mice

Despite the relative mildness of the CTL-induced hepatitis in these animals, and the fact that only a minor population of hepatocytes was destroyed during the course of the disease, all traces of viral RNA, replicative DNA intermediates, and nucleocapsid protein (HBcAg) were eliminated from the liver and serum following the administration of several different CTL clones. These effects

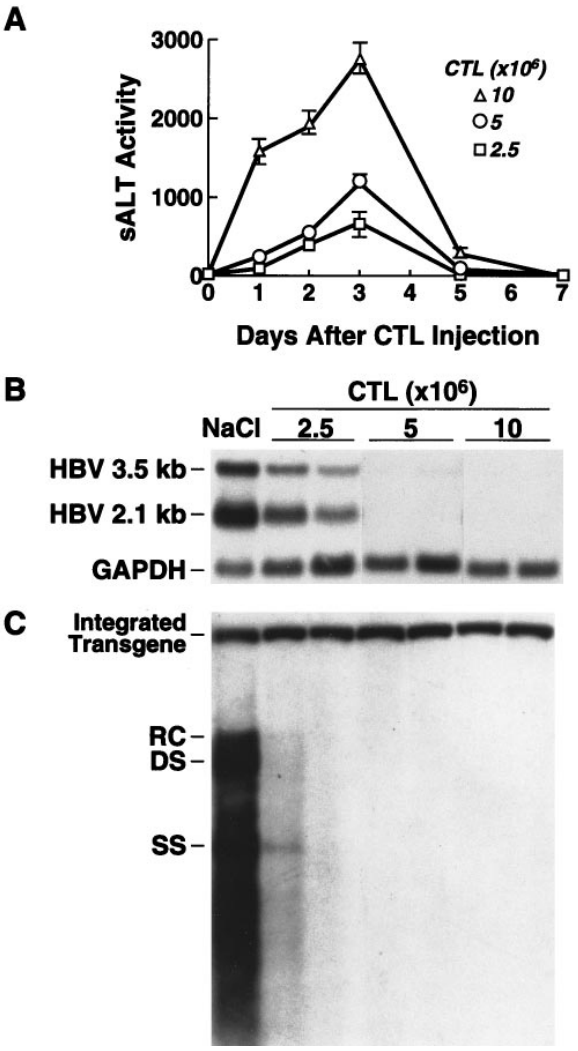

Figure 1. CTL-Mediated Liver Disease and Inhibition of HBV Gene Expression Replication: Dose Response

(A) sALT activity (± SEM) after intravenous injection of increasing numbers of HBsAg-specific CTL into groups of three mice from lineage 1.3.32 is expressed as units/liter. By comparing these values with the ALT profile obtained after injection of a known amount of liver extract, we estimated that no more than 6%, 10%, or 24% of the hepatocytes were killed following the injection of 2.5×10^6, 5×10^6, and 1×10^7 CTL, respectively (see Experimental Procedures).

(B) Effect of CTL on HBV gene expression in lineage 1.3.32. Dose response. Northern blot analysis of 20 μg of total liver RNA isolated from three groups of two mice 5 days after intravenous injection of increasing doses of CTL, as indicated. The steady-state HBV and GAPDH mRNA content was compared with total RNA pooled from 10 age-, sex-, and serum HBsAg-matched transgenic saline-injected controls, as indicated.

(C) Effect of CTL on HBV replication in lineage 1.3.32. Southern blot analysis of 20 μg of total liver DNA isolated from the same mice. HBV DNA content was compared with total DNA pooled from the same controls described above. All DNA samples were RNAse treated before gel electrophoresis. Bands corresponding to the expected size of the integrated transgene, relaxed circular (RC), double-stranded linear (DS), and single-stranded (SS) HBV DNA are indicated. Bands corresponding to the integrated transgenes can be used to normalize the amount of DNA bound to the membrane. The filters were hybridized with a ^{32}P-labeled HBV-specific DNA probe.

were dose dependent and displayed a characteristic sequential temporal profile.

CTL Dose Response

In this study, nine age-, sex-, and serum HBsAg-matched transgenic mice from lineage 1.3.32 (three mice per group) were injected intravenously with 2.5×10^6, 5×10^6, and 1×10^7 HBsAg-specific CTL (clone 6C2), and livers were harvested 5 days later. Histological analysis revealed that the vast majority of hepatocytes were cytologically normal in all CTL-treated livers at this time-point (see below), in parallel with the return of serum transaminase activity to normal levels (Figure 1A).

To examine the effect of CTL dose on HBV gene expression, Northern blot analysis was performed on total hepatic RNA. The most abundant viral transcripts in the liver of these animals are 3.5 and 2.1 kb in length (Guidotti et al., 1995). The 3.5 kb HBV transcript serves as the messenger RNA for the viral nucleocapsid proteins (HBcAg and HBeAg) and for the viral polymerase protein, and it functions as the viral pregenome since it is reverse transcribed by the polymerase to produce the first strand of viral DNA during viral replication. The 2.1 kb transcript serves as the messenger RNA for the viral middle and major envelope proteins (reviewed by Ganem and Varmus, 1987). Virtually all of the hepatocytes in these animals express viral RNA and nuclear HBcAg. Importantly, the 3.5 kb viral mRNA is most abundant in the centrilobular hepatocytes, corresponding with the centrilobular localization of cytoplasmic HBcAg particles and viral replicative intermediates in these livers (Guidotti et al., 1995). Centilobular zonation of liver gene expression is not uncommon (Lamars et al., 1989) and is thought to reflect the zonation of transcription factors in the hepatic lobule (Nagy et al., 1994).

The hepatic steady-state HBV and glyceraldehyde-3-phosphate (GAPDH) mRNA content of CTL-injected mice was compared with total RNA pooled from 10 age-, sex-, and serum HBsAg-matched transgenic controls injected with saline. As shown in Figure 1B for two representative mice per group, the hepatic steady state content of the 3.5 and 2.1 kb HBV mRNA were completely abolished in mice that received 5×10^6 and 1×10^7 CTL, and they were reduced 2- to 3-fold in mice that received 2.5×10^6 CTL. Note that virtually 100% of the hepatocytes express the viral RNA in these animals (Guidotti et al., 1995), yet it totally disappeared when only 10% of the hepatocytes were killed by the CTL (Figure 1A), suggesting that noncytolytic mechanisms contributed substantially to this effect.

To evaluate the effect of CTL dose on HBV replication, Southern blot analysis was performed on total hepatic DNA extracted from the same livers, and HBV DNA content was compared with total DNA pooled from the same controls described immediately above. Two representative mice per group are illustrated in Figure 1C. Hepatic HBV DNA replicative forms were undetectable in all the CTL-treated animals, including the mice that received the lowest CTL dose, thus suggesting that the viral replicative DNA intermediates are more responsive to elimination by the CTL than is the viral RNA. Note that viral replication is detectable in at least 30% of the hepatocytes in these mice (Guidotti et al., 1995), yet it was completely abolished when only 6% of the hepatocytes were killed by the CTL (Figure 1A), and most of these hepatocytes were located in the periphery of the hepatic lobule near their site of entry into the liver, not in the center of the lobule where replication is most active (see below), again suggesting that noncytolytic processes cause this effect.

To confirm that HBV mRNA disappears completely from all HBV RNA-positive hepatocytes following CTL administration, we performed in situ hybridization analysis on liver sections from CTL-injected and uninjected transgenic mice using an antisense ^{33}P-labeled HBV riboprobe that can detect all the overlapping HBV mRNAs. As shown in Figure 2 (left), lineage 1.3.32 (and 1.3.46, data not shown) expresses HBV mRNA in the vast majority of the hepatocytes. However, HBV mRNA was undetectable 5 days after the administration of 10^7 CTL in all of the hepatocytes (Figure 2, right). Consistent with this observation, nuclear and cytoplasmic HBcAg also completely disappeared from the liver within 5 days after injection of the CTL (see below).

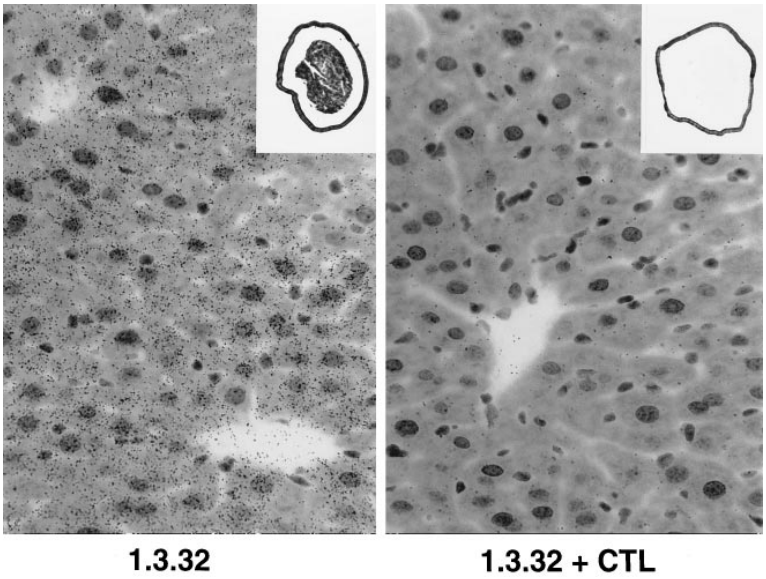

Figure 2. The CTL-Dependent Down-Regulatory Effect on HBV mRNA Extends to All HBV RNA-Positive Hepatocytes and to All HBV mRNA Species in Lineage 1.3.32

Detection of HBV mRNAs by in situ hybridization analysis on liver sections from transgenic mice intravenously injected 5 days earlier either with saline (left), or with 10^7 HBsAg-specific CTL (right). An anti-sense ^{33}P-labeled HBV riboprobe that can detect all the overlapping HBV mRNAs was used. (Hematoxylin and eosin; original magnification, 200×). Cross-sections of the large median lobe from the indicated mice were placed on film overnight and the derivative autoradiographs are shown (insets).

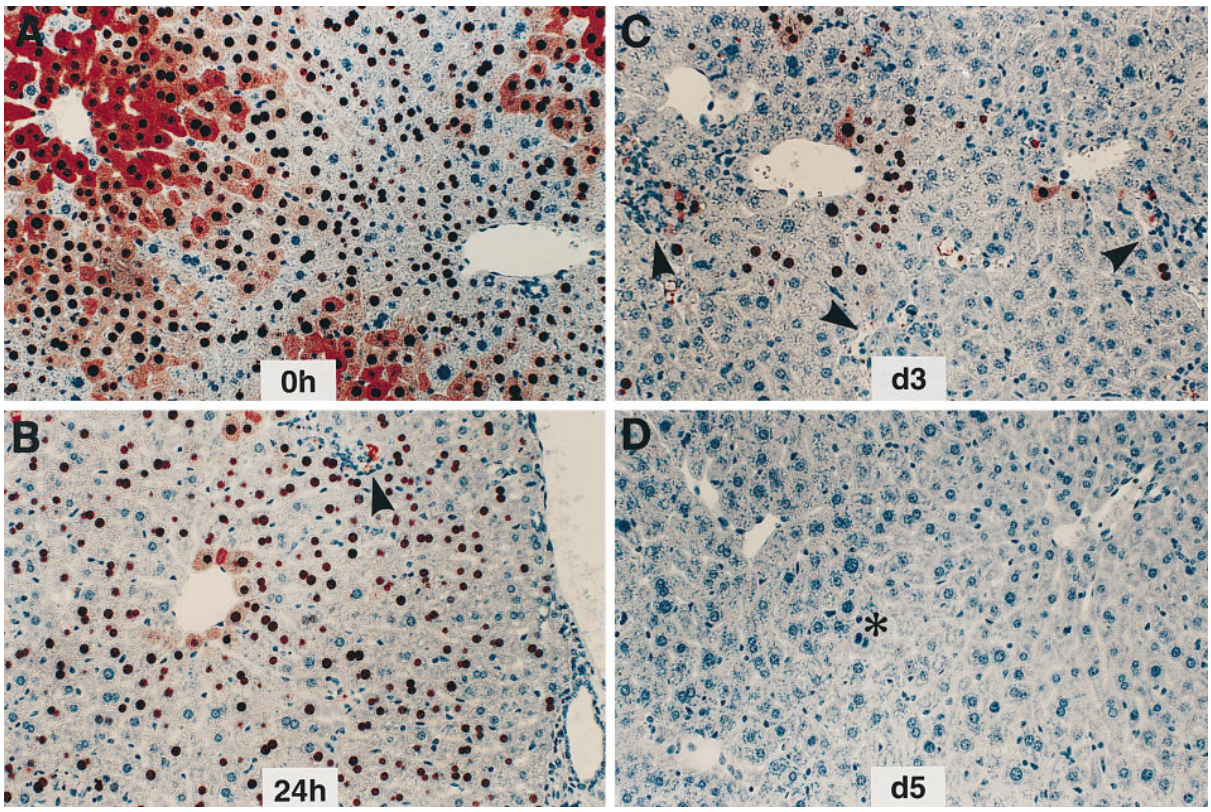

Figure 3. Effect of CTL on Hepatocellular HBcAg

Time course. Age-, sex-, and serum HBsAg-matched transgenic mice (18) from lineage 1.3.32 were injected intravenously with 10^7 HBsAg-specific CTL (clone 6C2), and livers were harvested at various timepoints thereafter and stained to detect HBcAg. Necroinflammatory foci are indicated with arrowheads and mitotically active hepatocytes with asterisks. (Immunoperoxidase stain for HBc/eAg; original magnification, 200×).

Time Course

The foregoing events occurred in phases, first affecting the cytoplasmic HBcAg, which became undetectable in centrilobular hepatocytes within 24 hr of CTL administration, in the absence of centrilobular liver disease, as shown in mice that received 1×10^7 CTL (Figure 3B). By day 3, at the peak of liver disease, scattered necroinflammatory foci were detectable in the hepatic lobule, especially in the periphery (Figure 3C, arrowheads), and nuclear HBcAg was profoundly reduced throughout the liver (Figure 3C). By day 5, HBcAg decreased to undetectable levels throughout the liver, despite the fact that the vast majority of the hepatocytes were cytologically normal (Figure 3D). HBcAg remained undetectable beyond day 14 and began to reappear, particularly in the cytoplasm of centrilobular hepatocytes, by day 28 (data not shown). Similar results were obtained by administration of 1×10^7 HBsAg-specific CTL in lineage 1.3.46 (data not shown).

To evaluate the extent to which hepatocyte turnover might contribute to this effect, we monitored the same liver sections for hepatocellular expression of proliferating cell nuclear antigen (PCNA) as a marker of cells in S phase (Kurki et al., 1988) (data not shown). A slight increase in the number of PCNA-positive hepatocytes was first observed 24 hr after CTL injection. At the peak of the liver disease (day 3) approximately 10% of the hepatocytes displayed PCNA-positive nuclei, subsiding to less than 1% at day 5 and baseline (less than 0.1%) at day 14. The relatively minor degree of liver cell regeneration seen in these animals is consistent with the relatively small number of hepatocytes that were killed by the CTL.

As shown in Figure 4B, HBV DNA replicative forms disappeared from the liver concomitant with the disappearance of cytoplasmic HBcAg (see Figure 3B), approximately 2 days before any change in the steady-state content of the 3.5 kb pregenomic RNA was detectable (Figure 4A). Like the decrease in hepatic HBcAg content, the duration of this effect was prolonged. Indeed, hepatic replicative DNA intermediates were undetectable by Southern blot analysis 4 weeks after CTL injection in one mouse, while they were partially restored in the other animal sacrificed at this timepoint (Figure 4B). The same profound and prolonged effect was observed in lineage 1.3.46 following the administration of 5×10^6 HBsAg-specific CTL (data not shown). These results strongly suggest that the loss of replicative DNA intermediates was linked to the disappearance of nucleocapsid particles, within which replication occurs, rather than to a decrease in pregenomic RNA template.

The disappearance of viral RNA from the liver displayed very different kinetics. Northern blot analysis of

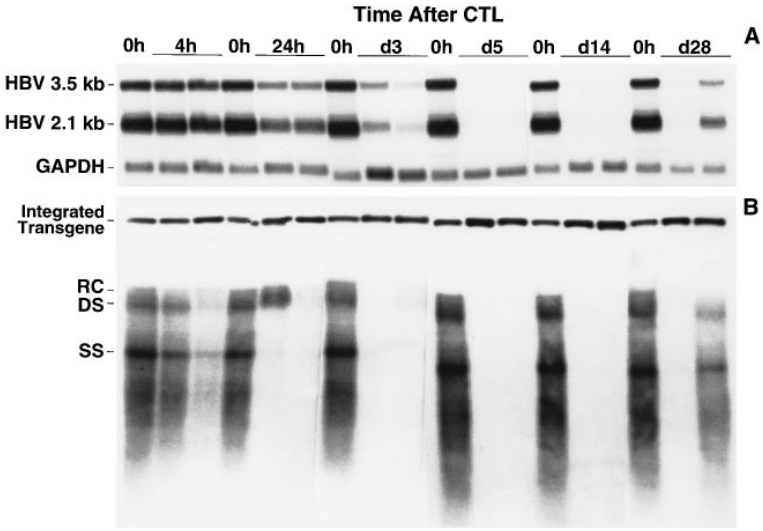

Figure 4. Effect of CTL on HBV Gene Expression and Replication in Lineage 1.3.32

Time course. Northern and Southern blot analysis was performed on total hepatic RNA and DNA extracted from the same mice described in the legend to Figure 3. Groups of two mice were sacrificed at 4 hr and 1, 2, 3, 5, 7, 10, 14, and 28 days after injection of 10^7 HBsAg-specific CTL.

(A) Northern blot analysis of 20 μg of total liver RNA isolated from mice sacrificed at the indicated timepoints. The steady-state HBV and GAPDH mRNA content was compared with total RNA pooled from 10 age-, sex-, and serum HBsAg-matched transgenic saline-injected controls.

(B) Southern blot analysis of 20 μg of total liver DNA isolated from the same mice. HBV DNA content was compared with total DNA pooled from the same controls described above. All DNA samples were RNAse treated before gel electrophoresis. The filters were hybridized with a ^{32}P-labeled HBV-specific DNA probe. DNA loading was normalized by comparing the signal intensity of the integrated transgene DNA seen at the top of each lane.

total hepatic RNA extracted from the same livers described above revealed that the hepatic steady-state content of the 3.5 and 2.1 kb HBV mRNA was unchanged 4, 24 (Figure 4A), and 48 hr (data not shown) after CTL injection. By day 3, however, HBV mRNA content decreased, reaching almost undetectable levels by day 5 when disease activity had nearly completely subsided. This profound down-regulatory effect involved both 3.5 and 2.1 kb HBV mRNAs and lasted beyond day 14 in all of the animals studied. At day 28, the suppressive effect of the CTL began to subside, and HBV gene expression started to reappear, particularly in one animal. Densitometric analysis of the autoradiographs (data not shown) revealed that the hepatic steady-state content of HBV mRNA of all the animals at days 5, 7, 10, and 14 was reduced by more than 95%, while the expression of housekeeping genes (e.g., GAPDH, rpL32, and β-actin), liver-specific transcription factors (HNF3α and HNF3β) and liver-specific genes (e.g., albumin and transthyretin) were quantitatively unaffected by the CTL (data not shown).

These changes in hepatic HBV gene expression and replication were reflected by a commensurate disappearance of circulating virions, measured as HBV DNA in pooled serum from groups of three mice at each timepoint (data not shown). Not surprisingly, serum HBV DNA content disappeared somewhat more slowly than the hepatic HBV DNA replicative intermediates. Concomitant with the reappearance of hepatic HBV DNA in one mouse 4 weeks after CTL administration (Figure 4), the serum HBV DNA content returned to preinjection levels at the same timepoint (data not shown).

HBsAg-Specific Polyclonal Cell Lines Also Down-Regulate HBV Gene Expression and Replication

To determine whether the CTL clones we used in this study reflect the functional potential of the HBsAg-specific CTL response in general, we monitored the effects of adoptively transferred HBsAg-specific polyclonal CTL lines in this system. Four age-, sex- and serum HBsAg-matched transgenic mice from lineage 1.3.32 were injected intravenously with 10^7 lymphocytes from a short-term polyclonal CTL line derived by 2 weeks of in vitro antigen stimulation of HBsAg-primed spleen cells from a B10.D2 mouse. The mice were bled at various timepoints thereafter to monitor sALT levels, and livers were harvested 7 days later. The polyclonal CTLs caused a modest elevation in sALT activity that was similar to the injection of 2.5×10^6 CTL clones (see Figure 1A) and subsided within 5 days (data not shown). Importantly, as illustrated in Figure 5B, HBV DNA replicative forms completely disappeared from the liver of these transgenic mice compared with saline-injected controls, as did hepatocellular cytoplasmic HBcAg (data not shown). Similar to the effect of 2.5×10^6 CTL clones (see Figure 1C), the CTL line was more effective at reducing hepatic HBV DNA replicative intermediates than HBV RNA (Figure 5A).

Perforinless CTLs Abolish HBV Replication without Killing the Hepatocytes

To evaluate the extent to which the antiviral effect of CTL reflects destruction of HBV-positive hepatocytes, we injected HBsAg-specific H-2^d-restricted $CD8^+$ CTL clones derived from HBsAg-primed perforin knockout mice (Kägi et al., 1994) into the HBV transgenic animals. These clones produce wild-type levels of IFNγ and TNFα mRNA following in vitro activation with plate-bound anti-CD3ε monoclonal antibody (MAb) (data not shown). Four age-, sex- and serum HBsAg-matched transgenic mice from lineage 1.3.32 were injected intravenously with 10^7 HBsAg-specific perforinless CTL (clone PKO-1), bled at various timepoints thereafter, and sacrificed 5 days later. As shown in Figure 6B, the perforinless CTL caused virtually no elevation in sALT activity compared with the injection of the same number of wild-type CTL

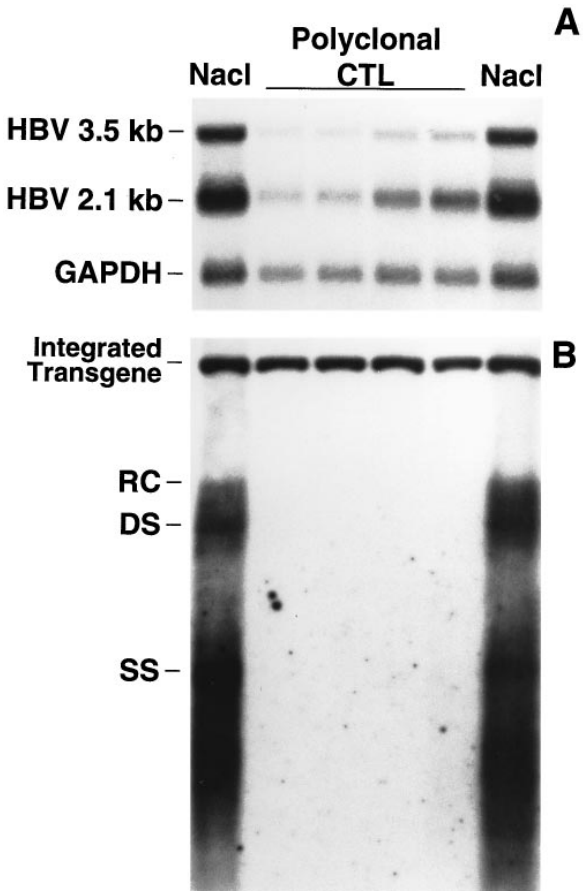

Figure 5. Effect of an HBV-Specific Polyclonal Cell Line on HBV Gene Expression and Replication in Lineage 1.3.32

(A) Northern blot analysis of 20 μg of total liver RNA isolated from four mice that were intravenously injected with an HBsAg-specific polyclonal CTL line (10^7 cells) and sacrificed 7 days later. The steady-state HBV and GAPDH mRNA content was compared with total RNA pooled from 10 age-, sex-, and serum HBsAg-matched transgenic saline-injected controls.

(B) Southern blot analysis of 20 μg of total liver DNA isolated from the same mice. HBV DNA content was compared with total DNA pooled from the same controls described above. All DNA samples were RNAse treated before gel electrophoresis. The filters were hybridized with a ^{32}P-labeled HBV-specific DNA probe. DNA loading was normalized by comparing the signal intensity of the integrated transgene DNA seen at the top of each lane.

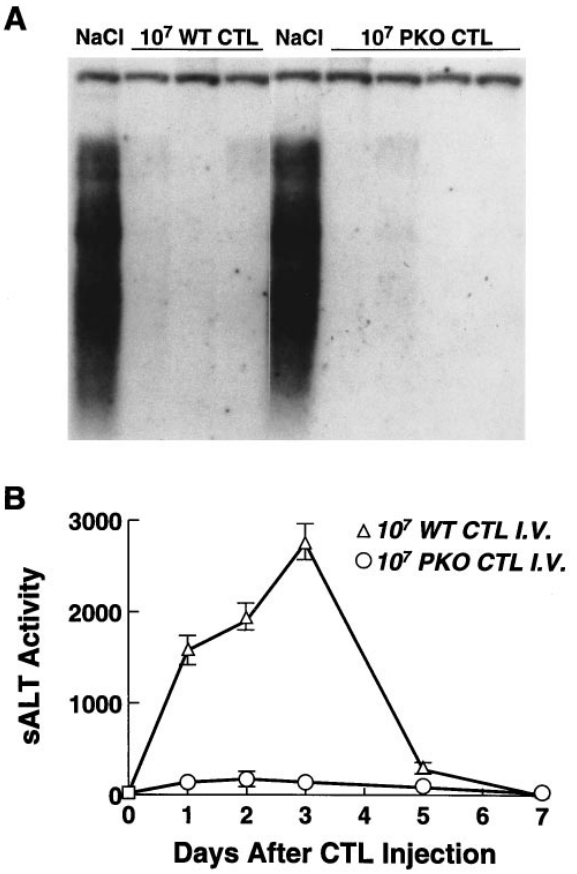

Figure 6. Effect of Perforinless HBV-Specific CTL on HBV Replication and Liver Disease in Lineage 1.3.32

(A) Southern blot analysis of 20 μg of total liver DNA isolated from four mice that were intravenously injected either with 10^7 wild-type CTL (clone 6C2) or with 10^7 perforinless HBV-specific CTL (clone PKO-1) and sacrificed 5 days later. HBV DNA content was compared with total DNA pooled from from 10 age-, sex-, and serum HBsAg-matched transgenic saline-injected controls. All DNA samples were RNAse treated before gel electrophoresis. The filters were hybridized with a ^{32}P-labeled HBV-specific DNA probe. DNA loading was normalized by comparing the signal intensity of the integrated transgene DNA seen at the top of each lane.

(B) sALT activity (± SEM) after intravenous injection of wild-type and perforinless HBV-specific CTL into the mice described above is expressed as units/liter.

(clone 6C2). Nevertheless, HBV DNA replicative forms completely vanished from the liver of these transgenic mice compared with saline-injected controls (Figure 6A), as did hepatocellular cytoplasmic HBcAg (data not shown), similar to the effect of wild-type CTL. This indicates that clearance of HBV DNA replicative forms from the liver does not require destruction of the hepatocyte.

Intrahepatic Cytokine Gene Expression in CTL Clones and Transgenic Mouse Liver after CTL Administration

All CTL clones used herein were found to produce IFNγ and TNFα mRNA and protein using a multiprobe RNase protection assay (Hobbs et al., 1993) and ELISA analysis (T. I. et al., unpublished data) following a 4 hr stimulation in vitro with plate-bound anti-CD3ε MAb (data not shown). To evaluate the magnitude and nature of the intrahepatic inflammatory response and the intrahepatic cytokine induction profile following CTL administration, we monitored CD3γ, CD4, CD8α, and cytokine mRNA expression in the transgenic livers at multiple timepoints following administration of clone 6C2. As shown in Figure 7, very low levels of CD3γ, CD4, CD8α, IL-1α, and IL-1β were the only mRNA species detected in livers from uninjected controls. Within 4–24 hr after CTL injection, however, the mRNA levels for these T cell markers (especially CD8) and IL-1 increased dramatically (Figure 7A), and IFNγ and TNFα mRNA appeared, despite little or no change in expression of other cytokines or a housekeeping gene, *rpL32*, that was used as an internal control (Figure 7B). The induced transcripts remained elevated for 72 hr and they decreased towards baseline levels by day 5 (Figure 7B).

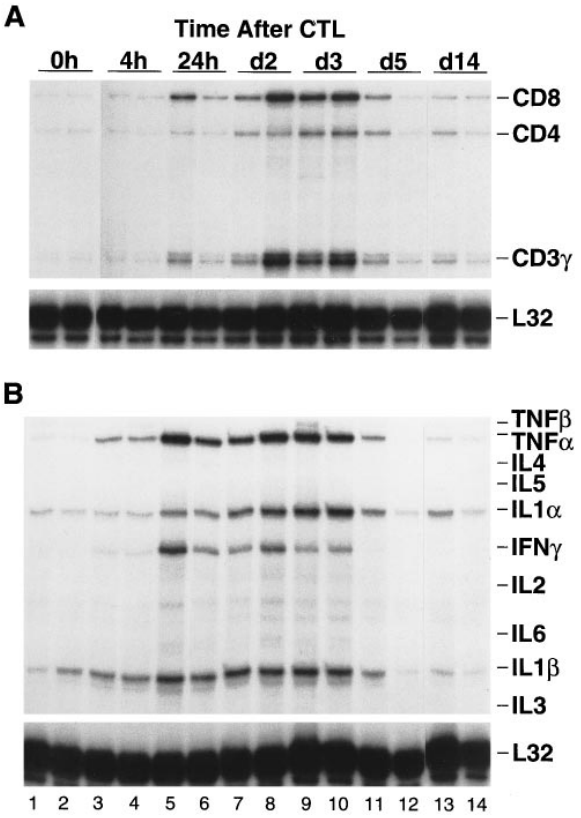

Figure 7. Analysis of Intrahepatic T Cell Markers and Cytokine mRNA Expression in Lineage 1.3.32 at Varying Intervals after CTL Administration

Total RNA (10 μg) extracted from the mice described in the legend to Figure 3 was analyzed by RNAse protection analysis for the expression of CD4, CD8, CD3γ, and assorted cytokines as indicated. Lanes 1 and 2 represent nontransgenic and transgenic resting livers, respectively. The remaining lanes show intrahepatic cytokine gene expression at varying times relative to CTL injection. The ribosomal protein light 32 (L32) was used to normalize the amount of RNA loaded in each lane.

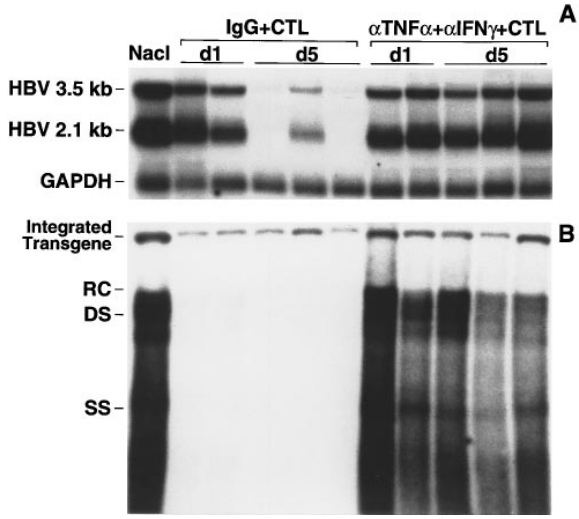

Figure 8. IFNγ and TNFα Mediate the Regulatory Effect of the CTLs

(A) Northern blot analysis of 20 μg of total liver RNA isolated from groups of 2–3 age-, sex-, and serum HBsAg-matched lineage 1.3.32 mice that were intraperitoneally injected with 250 μg of IFNγ and TNFα MAb 24 hr before the CTL and sacrificed either 1 or 5 days after CTL administration, as indicated. The latter group received a second injection of 250 μg of IFNγ and TNFα MAb 2 days after CTL injection. Control mice were simultanously injected with 250 μg of irrelevant hamster IgG. The steady-state HBV and GAPDH mRNA content was compared with total RNA pooled from 10 age-, sex-, and serum HBsAg-matched transgenic saline-injected controls.

(B) Southern blot analysis of 20 μg of total liver DNA isolated from the same mice. HBV DNA content was compared with total DNA pooled from the same controls described above. All DNA samples were RNAse treated before gel electrophoresis. The filters were hybridized with a ^{32}P-labeled HBV-specific DNA probe. DNA loading was normalized by comparing the signal intensity of the integrated transgene DNA seen at the top of each lane.

The kinetics and magnitude of these changes reflect the kinetics of CTL entry into the liver (maximal at 4 hr) and the recruitment of antigen-nonspecific inflammatory cells (maximal at 24–72 hr) (Ando et al., 1993). The induction of IFNγ and TNFα in the liver 4 hr after CTL injection (Figure 7B) coincides with the time when intrahepatic CTL concentration reaches a stable plateau (Ando et al., 1993). The minimal increase in T cell markers at this time (Figure 7A) confirms that very small numbers of CTL actually enter the liver relative to the number of HBV-positive hepatocytes (Ando et al., 1993). Nonetheless, these CTL are able to recruit a large number of host-derived inflammatory cells, indicated by a significant enhancement of T cell markers between 24–72 hr (Figure 7A), associated with further induction of inflammatory cytokines (Figure 7B).

CTL-Induced Suppression of HBV Gene Expression and Replication Is Mediated by IFNγ and TNFα

To determine the extent to which IFNγ and TNFα were responsible for the CTL-induced effects in lineage 1.3.32, we monitored the ability of MAbs specific for IFNγ and TNFα to modulate the cytopathic and regulatory effects of the CTL. Groups of mice were injected with IFNγ MAb or TNFα MAb or both at various times relative to the CTL, and were sacrificed 24 hr or 5 days after CTL administration. Control mice were injected with irrelevant hamster immunoglobulin g (IgG). Northern and Southern blot analysis was performed on total hepatic RNA and DNA extracted either from these animals and from saline-injected controls.

As shown in Figure 8, simultaneous administration of IFNγ and TNFα MAb completely blocked the CTL-induced inhibition of hepatic HBV replication and gene expression, and they also blocked the CTL-induced clearance of HBcAg from the hepatocytes in the same livers (data not shown). In contrast, each antibody was only partially effective when administered individually (data not shown), thereby suggesting that the two cytokines may activate independent regulatory pathways in the hepatocyte. Importantly, the antibodies did not influence the severity of the liver disease induced by the CTL in these animals (data not shown), providing additional evidence that the regulatory effect of CTL is not due to destruction of the hepatocytes.

Discussion

In this study, we demonstrate that HBV nucleocapsid particles, replicative DNA intermediates, and RNA can

be cleared from the liver of transgenic mice, and that HBV virions can be cleared from the blood, by a series of antiviral signals initially delivered by class I–restricted HBsAg-specific CTL. We also demonstrate that the antiviral effect of the CTL is mediated by at least two inflammatory cytokines and does not merely represent the destruction of HBsAg-positive hepatocytes. There are several reasons why we believe these claims are valid. First, clearance of viral nucleocapsids and replicative DNA intermediates begins prior to the onset of liver disease, and eventually involves every hepatocyte in these animals. Second, using wild-type CTL the disease is relatively mild, consisting of only small sparsely scattered necroinflammatory foci that kill a very small fraction of the hepatocytes, while all traces of the virus (except the integrated transgene) disappear from virtually 100% of the hepatocytes. Third, the cytopathic and regulatory effects of the CTL can be dissociated either by the administration of antibodies to IFNγ and TNFα, which completely abrogate the antiviral effect without reducing disease severity, or by administration of HBsAg-specific perforinless CTL, which completely abrogate HBV replication without causing liver disease. Additionally, in related experiments to be reported separately, we have shown that HBV gene expression and replication are abolished during persistent LCMV infection of the liver in response to the production of TNFα and type I interferons by LCMV-infected macrophages, in the absence of any evidence of liver disease (Guidotti et al., submitted).

The first detectable effect of the CTL on viral replication is the disappearance of cytoplasmic viral nucleocapsids and replicative DNA intermediates from the liver. The fact that these events occur prior to any decrease in viral RNA strongly suggests that at least two antiviral pathways are activated in the hepatocytes by the CTL: one that eliminates the nucleocapsids and their cargo of replicative DNA intermediates and another that down-regulates the RNA, which is transcribed from the integrated transgene. The similar kinetics with which the viral nucleocapsid particles and replicative DNA intermediates disappear from the cytoplasm suggest that these two effects are related and that they lead to the clearance of virions from the blood.

The simplest explanations for these observations are that the activated hepatocytes either prevent the formation of nucleocapsid particles or they accelerate their degradation, causing them to release their content of replicative intermediates into the cytoplasm, where they are destroyed by endogenous nucleases. Of course, it is also possible that the nucleocapsids are actively exported from the CTL-activated hepatocytes. This is unlikely, however, in view of the disappearance of viral DNA from the blood of the CTL-injected mice. Based on these considerations, plus the known stability of HBcAg particles in Xenopus oocytes (Zhou and Standring, 1992) and duck hepatocytes (Yu and Summers, 1994), we favor the degradation hypothesis, but additional experiments are needed to clarify this interesting observation.

The disappearance of the viral RNA from the liver following CTL administration also represents an important antiviral effect, since it precludes the production of new virus and the synthesis of supercoiled HBV DNA (Tuttleman et al., 1986), which is the normal transcriptional template of HBV during natural infection (see below). Indeed, the 3.5 kb pregenomic RNA serves multiple indispensable functions in the viral life cycle, including the production of the nucleocapsid particles and the replicative viral DNA forms that they contain (reviewed by Ganem and Varmus, 1987). Obviously, by virtue of this function alone it would not be surprising if the synthesis of new viral proteins and the assembly of new viral particles in an infected cell could be noncytolytically abolished by the CTL response.

The complete disappearance of the 2.1 kb envelope RNA further strengthens the antiviral potential of the CTL response in this model. We have previously reported that recombinant TNFα down-regulates the steady-state content of the 2.1 kb HBV envelope mRNA in the hepatocytes of other lineages of transgenic mice that only express this single gene product, and we have shown that it does so by posttranscriptionally destabilizing this viral transcript (Gilles et al., 1992; Guilhot et al., 1993). We have also found that the 2.1 kb HBV transcript is down-regulated in those animals, with delayed kinetics, following the administration of the same CTL that we used in the current study (Guidotti et al., 1994a). Recently, we have also shown that the CTL-induced disappearance of both the 2.1 and 3.5 kb HBV transcripts from the hepatocyte is primarly due to posttranscriptional mechanisms that are focused on viral sequences located between nucleotides 1239–3157 (Tsui et al., 1995).

Drawing upon the principles elucidated by this model, we propose that during HBV infection in man, virus-specific CTLs recognize HBV antigens on the surface of infected hepatocytes and perform two distinct functions. First, they kill a small fraction of infected hepatocytes, thereby causing the disease we recognize as viral hepatitis. Second, they secrete IFNγ and TNFα, thereby curing all of the hepatocytes by the intracellular inactivation pathways described above. The extent to which additional IFNγ and TNFα produced by the antigen-nonspecific inflammatory cells that the CTL recruit or activate in the liver contribute to this curative process remains to be determined. While it is clear that HBV is exquisitely sensitive to this effect, the demonstration of immunologically inducible intracellular inactivation pathways for HBV in the hepatocyte, does not imply that the same is true for all viruses and all cells. Although the literature suggests that many other viruses may also be susceptible to intracellular inactivation by the immune response, several factors must coexist for this process to occur. First, the local immune response must be vigorous enough to produce adequate amounts of specific cytokines. Second, the infected cell must be able to produce the appropriate antiviral activity in response to the cytokines. Third, the virus life cycle must include functions that are susceptible to those antiviral factors.

The ultimate impact of these cytokine-mediated effects on HBV gene expression and viral replication on the outcome of HBV infection is a question of considerable importance that remains to be resolved. Impinging

on this question are several aspects of HBV molecular and immunobiology, in addition to its susceptibility to cytokines, especially the involvement of hepatocellular destruction and regeneration to HBV clearance and the stability of the episomal HBV genome in the infected hepatocyte during an immune response. These issues merit discussion.

The hypothesis that the clearance of HBV from the liver during acute viral hepatitis requires the destruction of all of the infected cells should be considered in the context of two underappreciated aspects of HBV infection. First, HBV clearance occurs quite rapidly in most infected patients, as well as in woodchucks (Kajino et al., 1994) and ducks (Jilbert et al., 1992) that have been massively infected by their corresponding hepadnaviruses, without massive destruction of the liver. Second, the number of potentially infected hepatocytes in the human liver ($\sim 1 \times 10^{11}$) is not much less than the total body lymphocyte population ($\sim 1 \times 10^{12}$), and it is roughly equal to the total body CTL population, since only $\sim$25% of total lymphocytes are $CD8^+$. Therefore, rapid destruction of all infected hepatocytes would require the rapid commitment and deployment of an extraordinarily high proportion of the total body CTL into the liver. We view this to be an unlikely scenario, since HBV epitope-specific CTL precursor frequencies in the peripheral blood of acutely infected patients are usually in the 10^{-4} to 10^{-5} range (Chisari and Ferrari, 1995). The likelihood of this scenario is further diminished if one considers the constraints to CTL movement in a solid organ like the liver and the possibility that the greatly outnumbered CTLs may even be triggered to undergo apoptosis when antigen is presented to them by infected epithelial cells in the absence of strong costimulatory signals. Thus, the potential for a single CTL to kill many infected hepatocytes in vivo may be much less than the cytolytic capacity of the same CTL in vitro, where highly sensitive target cells are exposed to unphysiologically large numbers of effector cells in a cell pellet where there are no structural barriers to CTL movement. Additionally, in view of the rapidity of HBV clearance and the high proportion of infected hepatocytes in the liver, the cytodestructive CTL response should be regularly accompanied by fulminant hepatitis and death, and it is not.

Because the liver has extraordinary regenerative capacity, when some hepatocytes are destroyed other hepatocytes are triggered to divide (Michalopoulos, 1990). If HBV cannot survive in a regenerating hepatocyte, this process could also lead to viral clearance. Importantly, Fourel et al. (1994) have recently reported that clearance of persistent duck hepatitis B virus (DHBV) during antiviral therapy required drug toxicity, i.e., liver cell necrosis, inflammation, Kupffer cell activation, and hepatocyte turnover. While the authors emphasized the importance of hepatocellular regeneration for viral clearance in these studies, inflammation and macrophage (Kupffer cell) activation may have also contributed to this effect. In an independent study, the same investigators demonstrated that WHV clearance for the woodchuck liver is mostly independent of hepatocellular destruction and regeneration during massive acute WHV infection (Kajino et al., 1994), suggesting that other factors might play an important role in viral clearance in that model. It is noteworthy that in the present experiments, HBV gene expression and replication were completely abolished following the transfer of cytokine-producing perforinless CTL in the total absence of liver disease or hepatocellular regeneration, indicating that these HBV products can be eliminated from living nondividing hepatocytes.

Finally, it is important to emphasize that total clearance of HBV from infected cells requires more than inhibition of viral gene expression and replication. Absolute clearance requires elimination of the episomal covalently closed circular (ccc) HBV DNA species that serves as the viral transcriptional template in the nucleus of infected cells (Tuttleman et al., 1986). This aspect of the HBV life cycle cannot be approached in the HBV transgenic mouse model because, for unknown reasons, they do not produce ccc DNA (Guidotti et al., 1995). Thus, we cannot determine whether the ccc DNA is also susceptible to cytokine-mediated control in this model. However, since ccc DNA is abolished in the massive WHV infection model described above (Kajino et al., 1994) in the absence of massive destruction or regeneration of hepatocytes and in the presence of inflammatory cells and Kupffer cell hyperplasia, it is likely that ccc DNA can indeed be abolished from the resting hepatocyte by cytokine-dependent pathways like those described in the current study. If this is correct, the noncytolytic antiviral processes described in this paper are likely to play a central role in viral clearance during HBV infection. According to this scenario, viral clearance may be principally a CTL-inducible survival function of the infected cells rather than simply a destructive function of the immune response. If ccc DNA is not controllable by these mechanisms, they could paradoxically contribute to viral persistence by reducing the visibility of HBV by the immune response while leaving the viral transcriptional template in place.

Experimental Procedures

HBV Transgenic Mice

The HBV transgenic mice used in this study contain a terminally redundant copy of the complete HBV genome (ayw subtype) that extends from nucleotide 1068 just upstream of the viral enhancer I and X promoter completely around the viral genome to nucleotide 1982 just downstream of the unique polyadenylation site, as previously described (Guidotti et al., 1995). The hepatocytes from these animals replicate the virus at levels comparable to that seen in the infected livers of patients with chronic hepatitis without any evidence of cytopathology (Guidotti et al., 1995). Lineages 1.3.32 (official designation, Tg[HBV 1.3 genome]Chi32) and 1.3.46 (official designation, Tg[HBV 1.3 genome]Chi46) were expanded by repetitive back crossing against either the C57BL/6 or the B10.D2 parental strains, respectively, according to the genetic background of the founder. Lineage 1.3.32 mice were routinely backcrossed one generation against B10.D2 to produce H-$2^{b\times d}$ F1 hybrids prior to injection of B10.D2-derived polyclonal cell lines and CTLs. Weanlings were screened by analysis of serum for HBsAg and HBeAg using commercially available reagents (Abbott Laboratories, Abbott Park, Illinois) as previously described (Chisari et al., 1986), and 8- to 9-week-old males that were positive for both antigens were used for these studies.

Polyclonal T Cell Lines and CTL Clones

Several independently derived L^d-restricted $CD3^+$ $CD4^-$ $CD8^+$ CTL clones that recognize an epitope (IPQSLDSWWTSL) located between residues 28–39 of HBsAg, and secrete IFNγ and TNFα following antigen stimulation in vitro (Guidotti et al., 1994a) were used in this study. They were maintained by weekly restimulation with irradiated P815 cells that stably express the HBV large envelope protein (ayw subtype) containing HBsAg, as previously described (Ando et al., 1994). The CTL clones were washed, counted, suspended in HBSS containing 2% fetal calf serum 5 days after the last stimulation, and injected intravenously into the transgenic mice and into their nontransgenic littermates as controls.

CTL lines were derived from spleen cells from nontransgenic B10.D2 mice immunized and stimulated weekly as described (Ando et al., 1994). After 2 weeks of in vitro stimulation, the polyclonal cell lines were tested for Ag-specific cytotoxic activity, analyzed by flow cytometry (over 80% were $CD8^+$), washed, counted, suspended in HBSS containing 2% fetal calf serum, and injected intravenously into transgenic mice from lineage 1.3.32 and nontransgenic controls. Perforinless CTL were produced by repetitive immunization of perforin knockout mice (Kägi et al., 1994) with a recombinant vaccinia virus that expresses HBsAg, exactly as described for the foregoing CTL lines and clones. Following cloning by limiting dilution, an L^d-restricted HBsAg 28–39-specific $CD8^+$ IFNγ- and TNFα-producing CTL clone (PKO-1) that kills stably transfected P815 cells that express HBsAg (Ando et al., 1994) was selected for injection into the transgenic mice.

Anti-Cytokine MAbs

Hamster MAb H22 (endotoxin 0.25 EU/mg by the limulus amebocyte assay) and TN3 19.12 (0.5 EU/ml by the limulus amebocyte assay), specific for murine IFNγ and TNFα, respectively (Schreiber et al., 1985; Sheehan et al., 1989), were used in this study. Purified hamster IgG (Jackson Immune Research, West Grove, Pennsylvania) was used as a control antibody. All antibodies were diluted to a concentration of 1250 μg per ml with nonpyrogenic phosphate-buffered saline (GIBCO BRL, Gaithersburg, Maryland) immediately before injection. Antibodies (200 μl) were administered intraperitoneally 24 hr before and 2 days after the intravenous injection of the CTL.

Serological and Biochemical Analysis

Serum was examined for HBsAg, HBeAg, and anti-HBs titer using commercially available reagents (Abbott Laboratories) and, in selected cases, HBsAg and HBeAg were quantitated by comparison with known standards (Abbott Laboratories; Sorin, Saluggia, Italy) as previously described (Chisari et al., 1986). Serum DNAse-resistant HBV DNA was measured by dot-blot analysis exactly as decribed (Guidotti et al., 1995). Hepatocellular injury was monitored by measuring sALT activity (Chisari et al., 1989). Results were expressed as mean sALT activity ± SEM. To estimate the fraction of hepatocytes killed during the CTL-induced liver disease, we compared the sALT profiles of CTL-injected mice with the profile obtained after the intravenous injection of a known amount of solubilized liver extract prepared from an 8-week-old normal male mouse liver. To prepare the extract, the liver was homogenized by 10–20 strokes in a Potter–Elvehjem tissue grinder in ice-cold TE buffer (10 mM Tris–acetate [pH 8], 10 mM EDTA). Nonidet P-40 (Sigma Corporation, St. Louis, Missouri, final concentraion 1%) was added and after a 30 min incubation on ice the sample was centrifuged to remove the nuclei and cellular debris. Aliquots corresponding to 1/15 of the whole liver extract (6.7% of the liver) were injected intravenously into three nontransgenic mice and sALT activity was measured at timed intervals thereafter.

Histological Analysis

Tissue samples were fixed in 10% zinc-buffered formalin (Anatek, Limited, Battle Creek, Michigan), embedded in paraffin, sectioned (3 μm), and stained with hematoxylin and eosin as described (Chisari et al., 1989).

Immunohistochemical Analysis

The intracellular distribution of HBcAg and PCNA was assessed by the labeled avidin–biotin detection procedure as previously described (Guidotti et al., 1994c). In brief, paraffin-embedded sections in phosphate-buffered saline (PBS, pH 7.4) were treated for 10 min at 37°C with 3% hydrogen peroxide and washed with PBS. Sections were blocked with normal goat serum, and rabbit anti-HBc/eAg or mouse anti-PCNA (Dako, Carpinteria, California) primary antisera were applied at a 1:100 dilution for 60 min at 37°C (HBcAg) or overnight at 4°C (PCNA). After washing with PBS, a secondary antisera of biotin-conjugated goat anti-rabbit IgG $F(ab')_2$ (HBc/eAg) or biotin-conjugated goat anti-mouse IgG $F(ab')_2$ (Sigma Corporation, St. Louis, Missouri) were applied at a 1:100 dilution for 30 min at 37°C. The antibody-coated slides were washed with PBS, treated with streptavidin–horseradish peroxidase conjugate (extravidin, Sigma, St. Louis, Missouri) at a 1:600 dilution for 30 min at 37°C, stained with 3-amino-9-ethyl carbazole (Shandon-Lipshaw, Pittsburgh, Pennsylvania) (HBcAg) or 3,3′-diaminobenzidine tetrahydrochloride (Sigma) (PCNA) and counterstained with Mayer's hematoxylin (HBcAg) or nuclear Fast Red (PCNA) before mounting.

DNA Analysis

DNA Isolation and Southern Blot

Southern blot analysis was performed on total liver DNA from lineages 1.3.32 and 1.3.46 by agarose gel electrophoresis of 20 μg of restricted genomic DNA as previously described (Guidotti et al., 1995). Before electrophoresis, all DNA samples were digested with RNAse A (Boehringer Mannheim, Indianapolis, Indiana) at 10 μg/ml for 1 hr at 37°C. Nylon filters were hybridized with a HBV-specific ^{32}P-radiolabeled DNA probe as previously described (Guidotti et al., 1994b).

RNA Analysis

Northern Blot Analysis

Frozen tissues were mechanically pulverized and extracted by the acid–guanidium phenol–choloroform method (Chomczynski and Sacchi, 1987). Total RNA (20 μg) was analyzed for HBV, GAPDH, albumin expression by Northern Blot as previously described (Guidotti et al., 1994b).

RNase Protection Assay

The RNase protection assay for quantiation of mRNA was performed exactly as described (Hobbs et al., 1993). The mouse IL-1α (B), mIL-1β (A), mIL-2 (A), mIL-3 (B), mIL-4 (B), mIL-5 (C), mIL-6 (B), mIFNγ (B), mTNFα (A), mTNFβ (A), and mL32(A) subclones in pGEM-4 transcription vector were described in a previous report (Hobbs et al., 1993). The mCD4 (IC) (GenBank release 88; accession number M36850; nucleotides 233–523) and mCD3γ (IC) (accession number J03590; nucleotides 1643–1694 in exon 1, 42–65 in exon 2, and 42–166 in exon 3) subclones in pGEM-4 were prepared using polymerase chain reaction–assisted cloning (Hobbs et al., 1993). The mCD8α (DM) subclone in the pGEM-4 (which protects a 350 nt mRNA fragment) was provided by Dr. R. Hyman (The Salk Institute, La Jolla, California). The subclones were linearized with EcoRI and were used in template sets for T7 polymerase-directed synthesis of ^{32}P-labeled antisense RNA probes. The hybridization reactions, RNAse treatments, isolations of protected RNA duplexes, and resolution of protected probes by denaturing PAGE gels were as described (Hobbs et al., 1993). Dried gels were placed on film (XAR, Kodak) with intensifyng screens for various periods of time at −70°C.

In Situ Hybridization

This procedure was carried out exactly as described (Guidotti et al., 1995). The ^{33}P-labeled RNA probes used in this study span sequences on the noncoding and coding strand of HBV between residues 1243–1948 as previously described (Guidotti et al., 1995).

Protein Analysis

Immunoblot Analysis

Serum and organ homogenates were prepared as described (Chisari et al., 1986). HBV precore and core protein expression was detected by Western blot (immunoblotting) as previously described (Chisari et al., 1986). In brief, whole tissue powder was denatured in 100 mM Tris–acetate (pH 6.8), 2.5% SDS, boiled, centifuged, and total soluble protein was determined by Coomassie blue G-250 binding

(BioRad). Total liver protein extracts (100 μg) were separated by SDS 15% polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Amersham, Arlington Heights, Illinois). Filters were blocked with 5% nonfat dry milk (BioRad, Hercules, California) in TBS (50 mM Tris–acetate [pH 7.6] 150 m NaCl) for 2 hr at room temperature. To detect core/precore antigens, rabbit anti-HBc/eAg (Dako) primary antiserum was applied at a 1:500 dilution in TBS overnight at room temperature, washed three times for 15 min in TBS, and detected by incubation with a ^{125}I-labeled donkey anti-rabbit IgG (Amersham) for 1 hr at room temperature. Total liver protein extracts from nontransgenic littermates, and recombinant HBcAg (Sorin, Saluggia, Italy) were used as negative and positive controls, respectively.

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GenBank Accession Numbers

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Note Added in Proof

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