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Cytotoxic T lymphocytes inhibit hepatitis B virus gene expression by a noncytolytic mechanism in transgenic mice

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ABSTRACT **During hepatitis B virus (HBV) infection, distinct host–virus interactions may establish the patterns of viral clearance and persistence and the extent of virus-associated pathology. It is generally thought that HBV-specific class I-restricted cytotoxic T lymphocytes (CTLs) play a critical role in this process by destroying infected hepatocytes. This cytopathic mechanism, however, could be lethal if most of the hepatocytes are infected. In the current study, we demonstrate that class I-restricted HBV-specific CTLs profoundly suppress hepatocellular HBV gene expression in HBV transgenic mice by a noncytolytic process, the strength of which greatly exceeds the cytopathic effect of the CTLs in magnitude and duration. We also show that the regulatory effect of the CTLs is initially mediated by interferon γ and tumor necrosis factor α, is delayed in onset, and becomes independent of these cytokines shortly after it begins. The data indicate that the anti-viral CTL response activates a complex regulatory cascade that inhibits hepatocellular HBV gene expression without killing the cell. The extent to which this mechanism contributes to viral clearance or viral persistence during HBV infection remains to be determined.**

The control of hepatitis B virus (HBV) infection is thought to be mediated by the class I-restricted cytotoxic T-lymphocyte (CTL) response. Patients with acute viral hepatitis, who successfully clear the virus, mount a multispecific polyclonal CTL response to several HBV-encoded antigens, whereas persistently infected patients with chronic hepatitis do not (1). Hepatitis B surface antigen (HBsAg)-specific murine CTL clones cause a necroinflammatory liver disease when they are injected into HBsAg-positive transgenic mice, and the cytopathic effect of the CTLs is largely mediated by the inflammatory cytokines that they release when they are activated by antigen recognition (2, 3). While the data indicate that the CTL response to HBV plays a critical role in viral clearance and disease pathogenesis, the extent to which viral clearance depends on the destruction of infected cells is not known at this time.

We have shown that hepatocellular HBV gene expression in transgenic mice is negatively regulated, noncytopathically, by the pharmacological administration of recombinant interleukin (IL) 2 and tumor necrosis factor (TNF) α and that the IL-2 effect is mediated by TNF-α, which inhibits HBV gene expression by a posttranscriptional mechanism in these animals (4–6). We now report that hepatocellular HBV gene expression is profoundly inhibited, noncytopathically, by HBsAg-specific class I-restricted CTLs activated physiologically by antigen recognition *in vivo*, and we show that the inhibitory effect of the CTLs, which is mediated by TNF-α and interferon γ (IFN-γ), greatly exceeds their cytopathic effect in magnitude and duration.

MATERIALS AND METHODS

HBV Transgenic Mice. The two lineages studied were selected because they do not develop evidence of spontaneous liver disease and they display lineage-specific differences in hepatocellular HBsAg expression that permit distinction between cytolytic and noncytolytic aspects of the class I-restricted CTL–target-cell interaction *in vivo*. Lineage pFC80-219 (official designation Tg[HBs,HBV]Chi219) (inbred C57BL/6, H-2b) has been described (4–6). Since this lineage was produced by microinjection of inbred C57BL/6 embryos (H-2^b), the mice were routinely backcrossed one generation against B10.D2 to produce H-$2^{b\times d}$ F_1 hybrids prior to injection of the H-2^d-restricted CTLs. In selected experiments, the CTLs were also injected into inbred pFC80-219 (H-2^b) recipients to examine the role of antigen recognition in activation of the regulatory effect of the CTLs. Lineage 107-5D (official designation Tg[Alb-1,HBV]Bri66) (inbred B10.D2, H-2^d), which contains the entire HBV envelope coding region under the constitutive transcriptional control of the mouse albumin promoter (7), was also used in these studies.

Detection of HBsAg. The intrahepatic distribution of HBsAg was assessed by the indirect immunoperoxidase method, using 3-amino-9-ethyl carbazole (Shandon–Lipshaw, Pittsburgh) as a coloring substrate, as described (7). HBsAg was quantitated in serum and urine by a commercial radioimmunoassay (AusRIA; Abbott) as described (7).

***In Situ* Hybridization.** Snap-frozen liver sections, cut to 5- or 10-μm thickness, were transferred to polylysine-coated slides and fixed in 5% (wt/vol) paraformaldehyde/phosphate-buffered saline (PBS; 5 min at 0°C). The fixed sections were briefly washed in PBS (room temperature) and dehydrated by sequential transfer through 70% and 95% ethanol baths. *In situ* hybridization was carried out as described (8), using a ^{35}S-labeled 44-base probe that spans sequences on the noncoding strand of HBV between residues 1377 and 1420. After 4–8 weeks of exposure, the photoemulsion was developed and the sections were stained with hematoxylin/eosin.

HBsAg-Specific CTLs. Two independently derived L^d-restricted, $CD3^+$, $CD4^-$, $CD8^+$ CTL clones (designated 6C2 and D10) that recognize an epitope (IPQSLDSWWTSL) located between residues 28 and 39 of HBsAg and secrete IFN-γ after antigen stimulation *in vitro* (2, 3) were used in this

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Abbreviations: HBV, hepatitis B virus; CTL, cytotoxic T lymphocyte; HBsAg, hepatitis B surface antigen; IL, interleukin; TNF, tumor necrosis factor; IFN-γ, interferon γ; sALT, serum alanine aminotransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; m, murine; mAb, monoclonal antibody; EU, endotoxin unit(s).

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study. They were maintained by weekly restimulation with irradiated P815 cells that stably express the HBV large envelope protein (ayw subtype) containing HBsAg, as described (2). Five days after the last stimulation, the CTL clones were washed, and cells were counted, suspended in Hanks' balanced salt solution (HBSS) containing 2% (vol/vol) fetal calf serum, and injected intravenously into transgenic and nontransgenic recipients. Hepatocellular injury was monitored by measuring serum alanine aminotransferase (sALT) activity (9). Results were expressed as sALT activity (mean ± SEM). Tissue samples were also fixed in 10% (wt/vol) zinc in buffered formalin (Anatech, Battle Creek, MI), embedded in paraffin, sectioned (3 μm), and stained with hematoxylin/eosin (9).

RNA Analysis. Frozen liver tissue was mechanically pulverized and total hepatic RNA was analyzed for HBV, albumin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin mRNA content by Northern blot (10). The RNase protection assay for quantitation of cytokine and L32 mRNA was performed exactly as described (11) using a pool of the *Eco*RI-linearized subclones as templates for T7 polymerase-directed synthesis of ^{32}P-labeled antisense RNA probes. The murine (m) IL-1α(B), mIL-1β(A), mIL-2(A), mIL-3(B), mIL-4(B), mIL-5(C), mIL-6(B), mIFN-γ(B), mTNF-α(A), mTNF-β(A), and mL32(a) subclones in the pGEM-4 transcription vector used for this study have been described (11).

Anti-Cytokine Monoclonal Antibodies (mAbs). Anti-cytokine mAbs (250 μg per mouse) were administered intraperitoneally 24 h before or after the CTLs. Hamster mAb H22 [0.25 endotoxin units (EU)/mg by the limulus amebocyte assay (LAL)] and hamster mAb TN3 19.12 [0.5 EU/ml (LAL)], specific for mIFN-γ and TNF-α, respectively (12, 13), and hamster mAb ALF 161 [0.16 EU/mg (LAL)], hamster mAb B 122 [<0.1 EU/mg (LAL)], and rat mAb GL113 [0.075 EU/mg (LAL)], specific for mIL-1α, mIL-1β, and mIL-6, respectively (14–16), were used in this study. Purified hamster IgG (Jackson ImmunoResearch) and purified rat IgG (Schering–Plough) were used as a control antibody. All antibodies were diluted to 250 mg/200 ml with nonpyrogenic PBS (GIBCO) just before administration.

RESULTS

Hepatic HBV Gene Expression in Transgenic Mice. Both of the transgenic lineages used in this study express the HBV 2.1-kb HBs mRNA under the control of the internal HBs promoter. Northern blot analysis reveals that this is the only HBV-derived mRNA in lineage pFC80-219 (4, 5). The transcript is detectable in almost all of the hepatocytes in this lineage by *in situ* hybridization (Fig. 1 *A* and *B*). Lineage 107-5 also produces a 2.4-kb mRNA that is driven by the albumin promoter. HBsAg is detectable immunohistochemically in virtually 100% of the hepatocytes in this lineage (7). No significant variation in baseline expression of hepatic HBV steady-state mRNA was detected among heterozygous age-, sex- and serum HBsAg-matched transgenic offspring of either lineage.

HBsAg-Specific CTLs Cause a Necroinflammatory Liver Disease in HBsAg Transgenic Mice. Both CTL clones produce an HBsAg-specific necroinflammatory liver disease, the severity of which is lineage specific, when injected intravenously into these transgenic mice (3). The CTLs cause a relatively mild transient liver disease in lineage pFC80-219 that is detectable histopathologically (Fig. 1*C*) and biochemically as quantitative changes in sALT activity (Fig. 2*A*) within a few hours of CTL administration, reaches maximum severity by day 2, and subsides completely within a week. Histological analysis of the liver at the peak of the disease (Fig. 1*C*) demonstrates that the vast majority of the hepatocytes are normal but that there are occasional small widely separated necroinflammatory foci

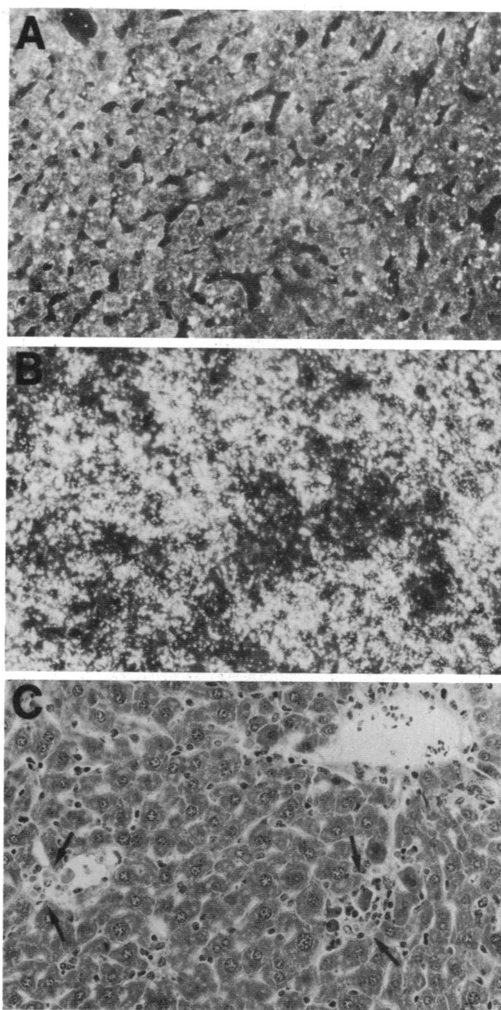

FIG. 1. Detection of specific HBV mRNA sequences by *in situ* hybridization with ^{35}S-labeled HBV DNA in the presence (*A*) or absence (*B*) of a 25-fold molar excess of unlabeled oligonucleotide probe. (Dark-field photomicrographs; ×160.) Histopathological analysis of transgenic mouse liver on day 2 after CTL administration at the peak of the disease (*C*) showing that the vast majority of the hepatocytes are cytologically normal. Two small necroinflammatory foci (arrows) are shown. (Hematoxylin/eosin; ×160.)

containing dead and degenerating hepatocytes that display the cytological changes characteristic of apoptosis (3, 17). By quantitative morphometric analysis of the number of dead and degenerating hepatocytes at the peak of the disease, we estimate that <5% of hepatocytes are destroyed by the CTLs in these animals (data not shown). In contrast, the same CTLs cause a much more severe disease in lineage 107-5, with 10-fold higher sALT levels (Fig. 2*A*), destruction of >50% of the hepatocytes, and a mortality rate between 40 and 50% (3). The extreme severity of the disease in this lineage is due to the retention of HBsAg filaments within the endoplasmic reticulum of the hepatocytes, which sensitizes them to the destructive effects of IFN-γ released by the CTLs after antigen recognition (3, 18).

HBsAg-Specific CTLs Inhibit Hepatocellular HBV Gene Expression in HBsAg Transgenic Mice. Despite the major differences in CTL-induced disease severity in the two lineages, they both displayed the same profound reduction in hepatic HBV mRNA content when examined 5 days after CTL administration (Fig. 2*B*). This strongly suggests that the regulatory effect of the CTLs is not merely due to destruction of the target hepatocytes. The regulatory effect of the CTLs appears to be relatively specific for HBV since we did not

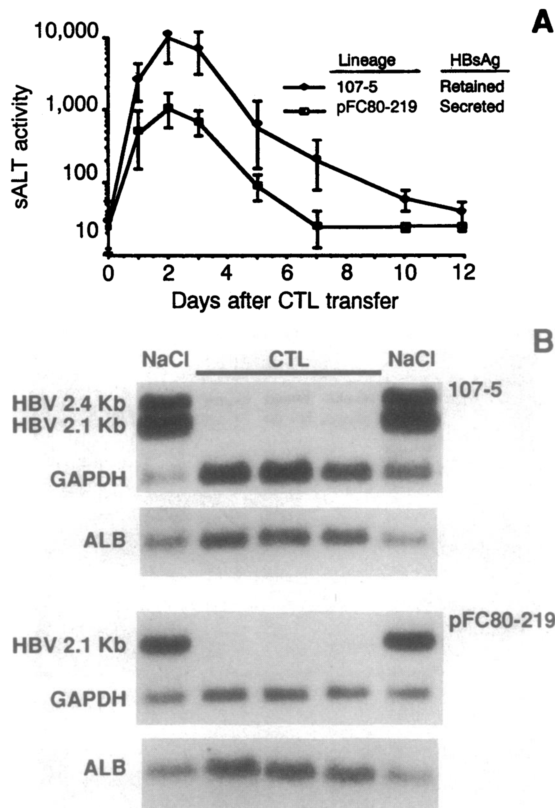

FIG. 2. (A) sALT activity after intravenous injection of 1 × 10^7 HBsAg-specific CTLs (clone 6C2) into groups of six mice from lineages pFC80-219 and 107-5 is expressed as units/liter. (B) Northern blot analysis of 20 μg of total liver RNA isolated from groups of three mice from each lineage 5 days after a single intravenous injection of 1 × 10^7 HBsAg-specific CTLs. ^{32}P-labeled DNA probes specific for HBV, GAPDH, and albumin (ALB) were used. Total RNA pooled from saline-treated mice was used as control.

observe any decrease in the hepatic steady-state content of GAPDH, albumin (Fig. 2B), or β-actin (data not shown) mRNA at this or any other time when HBV mRNA content was reduced (Fig. 3). Furthermore, the regulatory effect required antigen recognition by the CTLs, since it was not observed in inbred (H-2^b) transgenic mice from lineage pFC80-219 that lack the H-2^d restriction element utilized by the CTLs (data not shown). Although subsequent studies were performed in parallel in both lineages, with qualitatively similar results, the remainder of this report will focus principally on lineage pFC80-219.

Kinetics of the Regulatory Effect of the CTLs. *Hepatic HBV mRNA content.* Thirty age-, sex-, and serum HBsAg-matched transgenic mice from lineage pFC80-219 were injected intravenously with 1 × 10^7 HBsAg-specific CTLs (clone 6C2), and livers were harvested at various times for Northern blot analysis. Total RNA pooled from 20 saline-treated mice was used as a control. The steady-state hepatic content of the 2.1-kb HBV mRNA was unchanged when examined 1, 4, 12, 24, and 48 h after CTL injection during the period of maximum disease severity. By day 3, however, HBV gene expression decreased to nearly undetectable levels, and by day 5, when disease activity had nearly completely subsided, the HBV transcripts were barely detectable in all of the animals studied. Between days 7 and 10, the suppressive effect of the CTLs began to subside, and HBV gene expression returned to baseline at 10–14 days. Densitometric analysis of the autoradiographs (data not shown) revealed that the hepatic steady-state content of HBV mRNA of all the animals at days 3 and 5 was reduced by >95%, while the expression of housekeeping genes (e.g., GAPDH and β-actin) and liver-specific genes that are negatively regulated during the hepatic acute-phase response (e.g., albumin) was quantitatively unaffected by the CTLs.

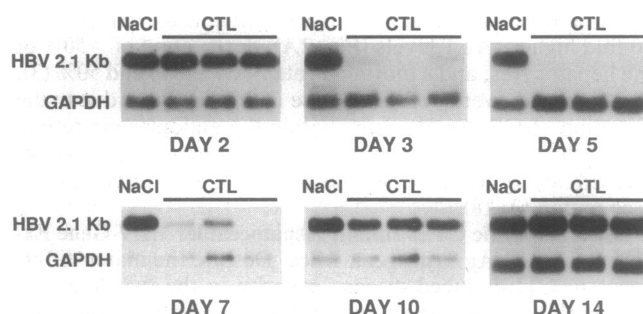

FIG. 3. Kinetics of the regulatory effect of the CTLs on hepatic HBV mRNA content in lineage pFC80-219. Northern blot analysis of 20 μg of total liver RNA isolated 2, 3, 5, 7, 10, or 14 days after groups of three transgenic mice received a single intravenous injection of 1 × 10^7 HBsAg-specific CTLs.

Serum HBsAg concentration. To determine whether the reduced hepatic HBV mRNA content was reflected at the protein level, we measured the HBsAg content of the liver and serum of CTL-treated and untreated mice from lineage pFC80-219. The liver was immunohistochemically negative, as reported (4). As shown in Fig. 4A, however, the serum HBsAg concentration fell in parallel with, and to a similar degree as, the decrease in the hepatic 2.1-kb HBV mRNA, but with slightly delayed kinetics. Presumably, this reflects the biphasic clearance of HBsAg that we observed after the intravenous injection of HBsAg-positive serum into nontransgenic littermates (Fig. 4B).

Cytokine Gene Expression in CTL Clones and in Transgenic Liver After CTL Administration. As a first step to determine whether cytokines contribute to the CTL-induced reduction in HBV gene expression, we used a multiprobe RNase protection assay to analyze the levels of IL-1α, IL-1β, IL-2,

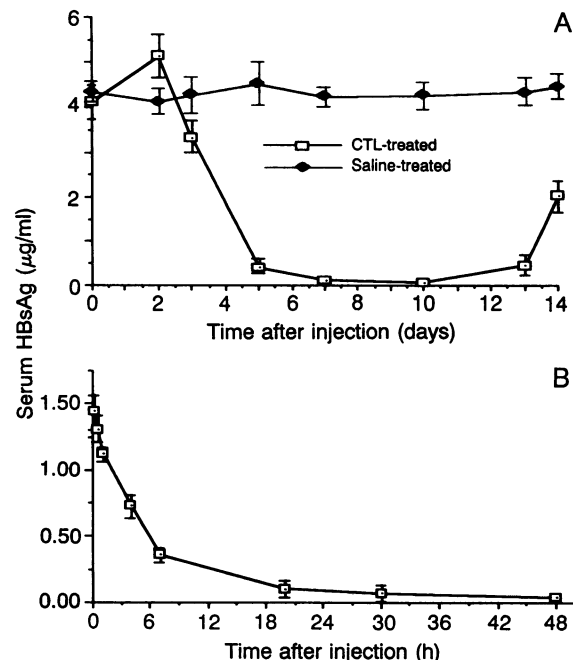

FIG. 4. (A) Kinetics of the regulatory effect of 10^7 CTLs on serum HBsAg concentration in groups of three mice from lineage pFC80-219. (B) Serum HBsAg half-life. Three nontransgenic mice were injected intravenously with 400 μl of HBsAg-positive serum from lineage pFC80-219 transgenic littermates and bled for HBsAg analysis 10 min, 30 min, and 1, 4, 7, 20, 30, and 48 h thereafter.

IL-3, IL-4, IL-5, IL-6, IFN-γ, TNF-α, and TNF-β mRNA expression by a panel of six HBsAg-specific CTL clones. After a 6-h stimulation *in vitro* with plate-bound anti-CD3ε mAbs, all clones were induced to produce IFN-γ, TNF-α, and to a lesser extent, TNF-β mRNAs, and we have shown (3) that the CTL clones secrete IFN-γ after antigen recognition *in vitro*. We then performed a time-course study of cytokine mRNA expression in transgenic liver after administration of clone 6C2. TNF-α, IL-1α, and IL-1β were the only mRNA species detected in livers from uninjected control transgenic and nontransgenic mice (Fig. 5). Within 1–4 h after injection of CTLs into transgenic mice, the mRNA levels for these three cytokines and for IFN-γ were clearly increased, despite little or no change in mRNA levels for the housekeeping gene, rpL32. All four transcripts remained elevated for 24 h after injection and then decreased to baseline levels by 72 h. No other cytokine mRNA species was evident in this analysis. The initial increase in cytokine mRNA coincided with the initial entry (1 h) and increase (4 h) in the number of intrahepatic CTLs (12), suggesting that the local production of the cytokines is a direct or indirect consequence of CTL activation.

CTL-Induced Suppression of HBV Gene Expression Is Mediated by IFN-γ and TNF-α. Administration of mAbs specific for IFN-γ or TNF-α 24 h before CTL injection either completely (IFN-γ) or nearly completely (TNF-α) blocked the CTL-induced inhibition of hepatic HBV gene expression monitored 5 days later (Fig. 6*A*), whereas antibodies to IL-1α, IL-1β, and IL-6 had no effect (data not shown). In the same study, we demonstrated that the mAbs had no effect on the severity of the disease induced by the CTLs in this lineage (Fig. 6*B*), demonstrating further that the regulatory effect of CTLs is not due to destruction of HBsAg-expressing hepatocytes in the livers of these mice. Importantly, the regulatory effect of the CTLs was not blocked when the IFN-γ- and TNF-α-specific mAbs were administered 24 h after injection of CTLs (Fig. 6*C*).

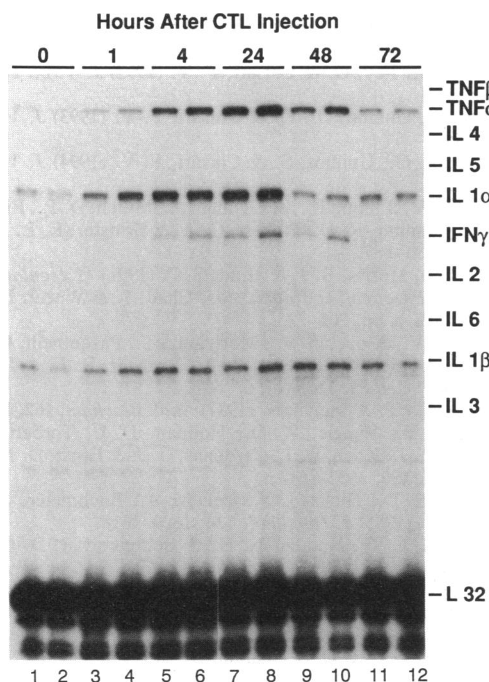

FIG. 5. Analysis of hepatic cytokine expression in lineage pFC80-219 0, 1, 4, 24, 48, and 72 h after CTL injection. Total RNA (10 μg) extracted from CTL-treated transgenic livers was assessed for the expression of assorted cytokines by RNase protection analysis. Lanes 1 and 2 represent uninjected nontransgenic and transgenic livers, respectively. The remaining lanes show hepatic cytokine expression of mouse transgenic livers at various times relative to CTL injection.

DISCUSSION

In this study, we have demonstrated that hepatocellular HBV gene expression is profoundly suppressed *in vivo* by noncytolytic regulatory signals delivered by class I-restricted HBsAg-specific CTLs and that the regulatory effect of the CTLs is initiated when they encounter antigen and release IFN-γ and/or TNF-α shortly after they enter the liver. Interestingly, the steady-state content of HBV mRNA and serum HBsAg levels do not begin to decline until 3 days later, suggesting that the cytokines activate a regulatory cascade within the liver and that other currently undefined factor(s) might deliver the final inhibitory signal to the hepatocyte. This concept is supported by the fact that the regulatory effect becomes independent of IFN-γ and TNF-α during the first 24 h after CTL administration, long before any change in HBV mRNA or HBsAg content is observed. Alternatively, these cytokines could directly deliver a regulatory signal to the hepatocyte that is not translated into decreased steady-state HBV mRNA content for 72 h.

Although we have previously demonstrated that the CTL clones cause a necroinflammatory liver disease in both of the

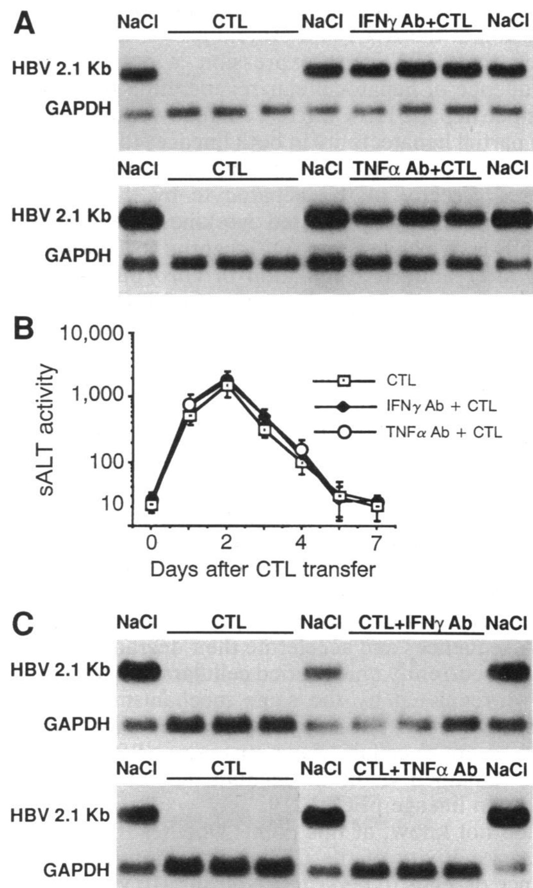

FIG. 6. IFN-γ and TNF-α mediate the regulatory effect of the CTLs. Northern blot analysis of 20 μg of total liver RNA isolated 5 days after CTL administration. Transgenic mice (three animals per group) were injected intraperitoneally with 250 μg of hamster mAbs specific either for IFN-γ or mTNF-α or with nonspecific hamster IgG 24 h before (*A*) or after (*C*) CTL administration. (*B*) Prior administration of antibodies to IFN-γ and TNF-α has no effect on the severity of the disease in lineage pFC80-219. sALT activity (in units/liter) was monitored daily after injection of CTLs in transgenic mice that had received either antibodies to IFN-γ or TNF-α or nonspecific hamster IgG 24 h earlier.

transgenic mouse lineages used in this study (2, 3), we believe that the regulatory effect of the CTLs does not merely represent the destruction of HBsAg-positive hepatocytes for several reasons. (*i*) The disease is relatively mild in lineage pFC80-219, consisting of only small sparsely scattered necroinflammatory foci that involve <5% of the hepatocytes, whereas the steady-state 2.1-kb HBV mRNA content of the liver is reduced by >95%. Since virtually all of the hepatocytes in this lineage express HBV RNA (refs. 4–6 and Fig. 1), the magnitude of the reduction in HBV gene expression is not compatible with the minimal amount of cell death in these animals. (*ii*) Even in lineage 107-5, which sustains an extremely severe liver disease and destruction of up to 50% of the hepatocytes, the reduction in 2.1- and 2.4-kb HBV mRNA content exceeds 95%, which is also out of proportion to the severity of the disease. (*iii*) It is possible to dissociate the cytopathic and regulatory effects of the CTLs in lineage pFC80-219 by the prior administration of antibodies to IFN-γ or TNF-α that completely abrogate the regulatory effect without reducing disease severity. (*iv*) The kinetics of the pathogenetic and regulatory effects of the CTLs are quite different—i.e., the peak of disease activity occurs on day 2, whereas maximal reduction of HBV mRNA occurs on or about day 5 after CTL administration. Based on these observations, we conclude that the CTLs deliver a noncytolytic signal to the hepatocytes that ultimately inhibits HBV gene expression at the RNA and protein levels. Additionally, we showed that HBV gene expression is not inhibited as a consequence of hepatocellular regeneration that follows injury, since hepatic HBV mRNA content was unchanged after a 70% partial hepatectomy in both lineages (data not shown).

The data also suggest that the regulatory signal is not delivered directly to the hepatocyte by the CTLs but is mediated indirectly by selected cytokines, specifically IFN-γ and TNF-α. We have shown (17) that the CTL clone secretes IFN-γ after antigen stimulation *in vitro* but, surprisingly, does not secrete a detectable amount of TNF-α. After CTL administration *in vivo*, the secreted IFN-γ may induce the secretion of TNF-α either by the CTLs themselves or by resident macrophages whose activation status is strongly suggested by the induction in the liver of monokines such as IL-1α and IL-1β (Fig. 5). This scenario is compatible with our previous observations that recombinant IL-2 down-regulates HBV gene expression posttranscriptionally (5) in the same lineage used in the current study and that it also does so by a TNF-α-dependent mechanism (6). This suggests that TNF-α may serve as a common mediator of the CTL and IL-2 effects and that they may be ultimately mediated by TNF-α-activated hepatocellular gene products that target HBV mRNA sequences and accelerate their degradation. Presumably other currently unidentified cellular transcripts may also be down-regulated by the same mechanism, but the data suggest that such putative cellular transcripts would not encode essential cellular functions, since 95% of the hepatocytes remained viable throughout the course of each experiment in lineage pFC80-219.

We do not know, at this point, whether the noncytolytic regulatory activity of the CTLs either contributes to viral clearance or leads to persistence during HBV infection. One might argue that suppression of viral envelope protein synthesis could interrupt virion assembly and, thereby, help to limit the spread of virus within the liver. Alternatively, this might represent a strategy adopted by the virus to escape detection by the immune system, in effect contributing to viral persistence.

In conclusion, the current observations document that physiologically activated immune effector cells release sufficient amounts of IFN-γ and TNF-α at the site of antigen recognition to effectively abrogate the expression of HBV gene products by the primary hepatocyte *in vivo*. These observations provide a glimpse into previously unapproachable host–virus interactions that could contribute to the pathogenesis of HBV-induced liver disease and the control of HBV gene expression by the hepatocyte. Additionally, they begin to define the extracellular and intracellular pathways that transmit a powerful regulatory message from the CTLs to the viral genome. These findings suggest directions for future investigation into the molecular and cellular determinants of clearance and persistence of this important human pathogen with the possibility that an understanding of these mechanisms may lead to the development of additional antiviral drugs.

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