Genomic analysis of the host response to hepatitis B virus infection

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Previous studies in hepatitis B virus (HBV)-infected humans and chimpanzees suggest that control of HBV infection involves the cells, effector functions, and molecular mediators of the immune response. The objective of the current study was to identify, in the liver of acutely HBV-infected chimpanzees, the spectrum of virus-induced and immune response-related genes that regulate the infection. The results demonstrate that HBV does not induce any genes during entry and expansion, suggesting it is a stealth virus early in the infection. In contrast, a large number of T cell-derived IFN- γ -regulated genes are induced in the liver during viral clearance, reflecting the impact of an adaptive T cell response that inhibits viral replication and kills infected cells, thereby terminating the infection.

The hepatitis B virus (HBV) is a noncytopathic hepatotropic DNA virus that causes acute chronic hepatitis and hepatocellular carcinoma (1). Viral clearance and disease pathogenesis during HBV infection are tightly associated with the appearance of a vigorous T cell response to all viral proteins (2, 3). In contrast, viral persistence and chronic hepatitis are associated with a markedly diminished HBV-specific T cell response (4, 5). CD8⁺ T cells are the main immune effector cells during HBV infection, because viral clearance and liver disease are blocked by depletion of CD8⁺ T cells in acutely infected chimpanzees (6). Furthermore, the onset of viral clearance in these animals is tightly associated with the appearance of virus-specific T cells (6) as well as CD3, CD8, and IFN- γ mRNA (6, 7) in the liver, indicating again that the adaptive T cell response plays a key role in this process.

Although cytolytic T cell functions certainly contribute to viral clearance, noncytolytic T cell functions also play a role, because adoptively transferred HBV-specific CD8⁺ T cells inhibit viral replication in HBV transgenic mice by a noncytopathic IFN- γ -mediated mechanism (8), and because HBV DNA largely disappears from the liver and blood long before the peak of liver disease in acutely HBV-infected chimpanzees (6, 7). Furthermore, IFN- α/β -mediated mechanisms also inhibit HBV replication noncytopathically in transgenic mice (9), and they do so by inhibiting viral capsid assembly (ref. 10 and S.W. and F.V.C., unpublished observations). Interestingly, genomic analysis of the livers and hepatocyte cell lines from those mice demonstrated a close association between the antiviral effects of both IFN- γ and IFN- α/β and the induction of hepatocellular genes that might mediate the antiviral effects (11). These genes include GTP-binding proteins (e.g., GBP-1 and TGTP) known to inhibit other viral infections (12, 13), as well as components of the immunoproteasome (LMP2, LMP7, and MECL-1), IFN-stimulated protein 15 (ISG15), ubiquitin-specific protease 18 (Usp18), the chemokine IP-10, and the signal transducer and activator of transcription (STAT)-1 (11).

The current study was prompted by the desire to validate these findings in the setting of an acute HBV infection where the genomic changes occurring during viral entry, spread, and clearance can be uniquely identified. We did so by serially profiling the liver transcriptome in three acutely HBV-infected chimpanzees, searching especially for two distinct groups of cellular genes: those whose expression might correlate with the entry and expansion of the virus that might reflect the innate immune response, and those whose

expression correlates with viral clearance reflecting the adaptive immune response that terminates infection.

Materials and Methods

Chimpanzees. Three healthy young adult HBV-seronegative chimpanzees (Ch1615, -1627, and -5835) were used in this study. The animals were handled according to humane use and care guidelines specified by the Animal Research Committees at the National Institutes of Health and The Scripps Research Institute. They were housed at Bioqual Laboratories (Rockville, MD), an American Association for Accreditation of Laboratory Animal Care International-accredited institution under contract to the National Institute of Allergy and Infectious Diseases. The animals were inoculated with 10^8 genome equivalents of a monoclonal HBV isolate (genotype *ayw*) contained in pooled serum from HBV transgenic mice (14). Before inoculation and weekly thereafter, blood was obtained by venipuncture and analyzed for serum alanine aminotransferase activity (sALT) as described (8). Six weeks after inoculation, Ch1615 and -1627 received three daily i.v. injections of either a humanized chimeric monoclonal anti-human CD4⁺ antibody (cM-T412) or an irrelevant control antibody, respectively, as described (6). The course of infection, inflammatory infiltrate, and kinetics of viral clearance were not affected in these animals (6). Liver biopsy and DNA and RNA preparation protocols are available in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

Gene Expression Analysis. At selected time points, 100 ng of total liver RNA isolated from frozen liver biopsies was used to prepare cRNA by the reduced-volume RNA amplification protocol described by Baugh *et al.* (15). The cRNA was hybridized to high-density oligonucleotide arrays (HG-U133A Human GeneChips, Affymetrix, Santa Clara, CA), which interrogate the expression of $\approx 22,000$ human genes. Primary image analysis was performed with GENECHIP VERSION 5.0 (Affymetrix). Relative gene expression levels were normalized throughout the entire data set by using algorithms in the DCHIP software package (16, 17). Genes not called “present” by GENECHIP at least at one time point were excluded from further analysis, and the remaining values were logarithm-transformed to base 10. Genes were identified whose expression correlated (by Pearson’s correlation) either with the pattern of log-transformed intrahepatic HBV DNA or with the expression profile of specific prototype cellular genes described in the text. A Pearson correlation coefficient of ≥ 0.7 over all time points in all three chimpanzees was required for a gene to be further analyzed. Finally, selected genes had to be called “present” at the peak of intrahepatic HBV DNA levels or at the expression maxima of specific prototype genes, and they had to be called “induced” by GENECHIP at the same time point

Abbreviations: HBV, hepatitis B virus; STAT, signal transducer and activator of transcription; Chn, chimpanzee *n*; HCV, hepatitis C virus; 2'5'OAS, 2'5' oligoadenylate synthetase; sALT, serum alanine aminotransferase activity; NK, natural killer; TCR, T cell antigen receptor.

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relative to the preinfection sample in all three animals. Genes were selected as “induced during viral clearance” if they were called “present” and “induced” relative to preinfection levels in weeks 12, 14, and 16 in Ch1627, 14 and 16 in Ch1615, and 14 and 18 in Ch5835. In addition, the expression level of these genes had to be higher during viral clearance than during the weeks before peak viremia in the same animal (i.e., weeks 4 and 6 in Ch1627 and 2 and 4 in Ch1615 and -5835). For Ch1627, HBV clearance-associated genes were grouped according to their induction kinetics into early, middle, and late genes based on the week (i.e., 12, 14, or 16) when peak induction was observed. Gene expression profiles were visualized by using GENESPRING VERSION 5.0 (Silicon Genetics, Redwood City, CA) by using gene expression values normalized to the 10th and 90th percentiles for each gene.

Results

Course of HBV Infection in Acutely Infected Chimpanzees. Ch1615, -1627, and -5835 were inoculated i.v. with 0.5 ml of HBV-transgenic mouse serum containing 1×10^8 genome equivalents of HBV DNA (6). As shown in Fig. 1, all three animals, including the CD4-depleted animal, became infected, developed acute hepatitis, and cleared the infection with remarkably similar kinetics (liver HBV DNA levels are shown in Table 2, which is published as supporting information on the PNAS web site). Viral spread in Ch1627 (Fig. 1A) and -5835 (Fig. 1C) was not accompanied by induction of CD3, IFN- γ , or 2'5' oligoadenylate synthetase (2'5'OAS) mRNA, which was monitored by RNase protection analysis (Fig. 1), suggesting that the virus was not recognized by the innate or adaptive immune response in those animals at that time. In contrast, CD3, IFN- γ , and 2'5'OAS mRNA were all transiently induced in Ch1615 during week 5, coinciding with a transient spike of elevated sALT activity (Fig. 1B). All of these events were abruptly terminated by antibody-mediated CD4 depletion during week 6 (Fig. 1B, vertical arrow), suggesting that they reflected the atypically early onset of an adaptive immune response in this animal. This notion is further supported by the observation that, several weeks later, viral clearance in all animals coincided with the appearance of CD3, IFN- γ , and 2'5'OAS mRNA (Fig. 1), elevated sALT (Fig. 1), and HBV-specific T cells in the liver (ref. 6 and data not shown). Collectively, these results suggest that these T cells and their products contributed to viral clearance in these animals. Importantly, in all three animals, the viral DNA decreased 10- to 50-fold in the liver before the number of hepatitis B core antigen-positive hepatocytes began to decrease (Fig. 1), indicating that the decrease in viral DNA did not reflect the destruction of infected cells.

Liver Gene Expression Profiles in Acutely Infected Chimpanzees. The stars in Fig. 1 indicate the time points when liver gene expression profiling was performed in the animals. In this study, we attempted to identify two classes of genes: one group whose expression correlated with hepatic viral DNA content and therefore could reflect the initial (innate) host response to HBV in the liver, and a second group whose expression correlated with viral clearance and who are likely to be induced by the adaptive immune response to the virus.

Liver Genes Induced by HBV. The first group of genes displayed expression patterns that correlated (directly or inversely) with the amount of HBV DNA in the liver over the entire time course profiled. To reduce the chance of misinterpreting random fluctuations in gene expression in individual animals, we calculated the Pearson correlation between the expression of each gene and the amount of HBV DNA at each time point in all three animals, and we restricted our focus to genes whose changing expression levels correlated with the changing viral DNA content in the liver with a correlation coefficient of at least 0.7. Importantly, as shown in Fig. 2A, no genes fulfilled these criteria. Furthermore, no transcripts were uniformly induced or repressed if we limited our focus solely

Fig. 1. Course of acute HBV infection in chimpanzees after experimental i.v. inoculation of 10^8 genome equivalents of HBV in week 0. (A) Ch1627 was also injected with an irrelevant control antibody in week 6. (B) Ch1615 was injected with a CD4-specific monoclonal antibody as described in the text. (C) Ch5835 did not receive any additional treatment. Intrahepatic HBV DNA levels (black squares) is expressed as a percentage (% max) of the peak HBV DNA levels in the liver of each animal (actual liver HBV DNA levels are presented in Table 2). Hepatitis B core antigen-positive (HbCag) hepatocytes (gray bars) are expressed as percentage of the total number of hepatocytes. sALT (open circles) is expressed in units per liter. Intrahepatic expression profiles for IFN- γ , CD3, and 2'5'OAS were determined by RNase protection assay. Vertical arrows indicate the time of antibody injection. Stars indicate the time points for which gene expression profiling was performed.

Fig. 2. Liver gene expression profile during acute HBV infection. (A) Genes correlated with viremia in all chimpanzees. No genes correlated positively or negatively with the level of intrahepatic HBV DNA during the time course of acute HBV infection in all three animals. (B) Liver gene expression profile associated with viral clearance in all three acutely HBV-infected chimpanzees. The gene identities are shown in Table 1 and the primary data in Tables 3 and 4. Green lines show the intrahepatic HBV DNA as a percentage (% max) of the corresponding peak levels. For optimal visualization, gene expression levels (red lines) are shown after normalization to the 10th and 90th percentiles for each gene. Values on the x axis represent weeks after inoculation with HBV.

to the lag phase of infection (weeks 0–2 in Ch1615 and -5835) or to the phase of logarithmic expansion (weeks 4–6 in Ch1615 and -5835 and weeks 4–7.5 in Ch1627) of the virus (data not shown). Because virtually 100% of the hepatocytes were infected in all three animals (see hepatitis B core antigen-positive hepatocytes in Fig. 1), the failure of the virus to induce cellular gene expression as it spread throughout the liver suggests that HBV does not induce an innate immune response at the site of infection.

Liver Genes Associated with Viral Clearance. To identify genes associated with viral clearance, we searched for transcripts that were either induced or suppressed when viral DNA disappeared from the liver. We identified 59 unique genes (“Selected as induced during viral clearance,” Table 1, and indicated with a Y in column 5 of Table 3, which is published as supporting information on the PNAS web site) that were induced during viral clearance in all three animals (average peak fold change, 10.7), and none that were suppressed. The expression profile of these genes reflects a type 1 T cell response, because it was heavily weighted toward genes expressed by α/β T cells (e.g., T cell receptor β), γ/δ T cells (e.g., T cell receptor γ), the cytolytic effector molecule granzyme A, and many genes known to be induced by IFN- γ , which itself correlated with viral clearance (Fig. 1). The IFN- γ -induced genes include *STAT1*, MHC class I, MHC class II, immunoproteasome subunits *LMP2*, *PA28 α* and - β , ubiquitin D, *E2L6*, tapasin, the chemokines

monokine induced by IFN- γ (*MIG*), *IP-10*, and *RANTES*, the GTPase *GBP2*, and several other IFN- γ -induced genes [e.g., *IFI27* (18) and *IFI30* (19), and tryptophanyl-tRNA synthetase (20)], whose relevance to the T cell response and viral clearance is not immediately apparent. IFN- γ itself, however, is not in this list because it was scored present in only a few samples (data not shown and Table 4, which is published as supporting information on the PNAS web site), suggesting that the absolute level of intrahepatic IFN- γ expression (Fig. 1) was too low to be consistently detected by the microarrays used. 2’5’OAS, which is induced during viral clearance (Fig. 1), also is not part of this list, because its induction during viral clearance in Ch1615 was very brief (Table 4) and therefore did not satisfy our selection criteria. In addition, several genes not known to be inducible by IFN- γ (e.g., apolipoprotein L3, butyrophilin A3, cathepsin C, cofilin 1, complement component C1q, galectin 3, lysozyme, *Plac8*, superoxide dismutase, and Ig heavy and light chain alleles) were also induced during viral clearance.

Next, to identify viral clearance-associated genes that might have been excluded by our stringent selection criteria, we searched for genes whose expression correlated with the profiles of prototype genes, specifically T cell antigen receptor (*TCR*) β , *TCR* γ , *CD3D*, *IFN- γ* , *MIG*, *RANTES*, *IP-10*, and *sALT*, as indicated in columns 6–13 of Table 3. This increased the number of genes induced during viral clearance to 110, all of which are shown in Fig. 2B and identified in Tables 1 and 3.

This list contains additional T cell (*CD38*, *CD53*, and granzyme A), natural killer (NK) cell (*CD53* and granzyme K), B cell (*CD48* and *CD83*), macrophage (*CD68*), chemokine (*CXCL11* and *CXCR4*), GTPase (*GBP1*), complement (*C3a*), IFN-stimulated (*ISG20* and *IFI16*), and immunoproteasome (*MECL-1*) genes that were not included in the first-pass analysis. Importantly, 84 of these 110 genes were closely correlated with the *TCR* β and - γ mRNA expression profiles (Table 3), 88 correlated with the induction profiles of *MIG*, *IP-10*, or *RANTES*, which are known to be highly induced by IFN- γ (21, 22), and 79 correlated with *sALT* levels in all three chimpanzees (Table 3 and Fig. 1). It is noteworthy that all of these genes returned to baseline when the viral DNA was eliminated (Fig. 2B). Because that correlation was not required for a gene to be designated as “clearance-associated,” it strongly supports the notion that these genes were functionally related to viral clearance.

Sequential Induction of Clearance-Associated Liver Genes in Ch1627.

Examination of the clearance-associated gene expression profile of Ch1627 (Figs. 2B and 3) reveals three distinct gene clusters whose expression peaked in week 12, 14, or 16 and were designated early, middle, and late clearance genes, accordingly (Fig. 3). The spacing of the sampled time points in the other chimpanzees did not permit sequential gene clusters to be identified in those animals. As shown in Table 1 and Fig. 3 (in blue), the early gene cluster consisted of 35 genes that included the *TCR* β locus, several IFN- γ -induced genes including MHC class I and II genes, the TAP-binding protein (tapasin), the immunoproteasome components *PA28 α* and - β , the chemokine *RANTES*, and IFN- γ -inducible protein 30, suggesting that they are mainly T cell-related and IFN- γ -induced genes that mediate antigen processing and presentation and inflammatory cell recruitment.

The middle gene cluster [Fig. 3 (shown in red) and Table 1] consisted of 73 genes, including *CD3D*, the *TCR* γ locus, a T cell activation marker (*CD38*), and the cytolytic effector granzymes A and K, as well as a T-LAK cell-originated protein kinase, a macrophage antigen (*CD68*), and a B cell membrane protein (*CD48*). Several additional IFN- γ -induced genes were also detected, including *STAT1*, the chemokines *IP-9*, *IP-10*, and *MIG*, the immunoproteasome component *MECL-1*, several ubiquitin-related genes, the GTPase *GBP1*, and its closely related family member GTPase guanylate-binding protein 2. Thus, the second wave of

Table 1. Liver genes associated with HBV clearance in all three chimpanzees

Gene/EST	Selected as induced during viral clearance	Peak expression* (Ch1627)	Induced during clearance in	
			HCV chimps [†]	HBVtg HCs [‡]
Adenosine deaminase		E		
Aldo-keto reduc. B1		M		
Annexin A2		E		
Apolipoprotein L 3	+	E		
ICB-1		M		
β -2-microglobulin		M		+
Butyrophilin 3 A3	+	M		
C3AR1		M		
Cat eye syndrome 1		M		
Cathepsin B		M		
Cathepsin Cd	+	M		
CD3- δ		M		
CD38		M		
CD48	+	M		
CD5	+	M	+	
CD53		M		
CD68		M		
CD74	+	E		
CD83	+	E		
CLN2	+	M		
Cofilin 1	+	E		
C1q	+	E	+	
CCL5 (RANTES)	+	E	+	
CXCL10 (IP-10)	+	M		+
CXCL11 (IP-9)		M		
CXCL9 (MIG)	+	M	+	+
CXCR4		M		
EMR1		M		
EST		E		
EST		E		
EST	+	M		
EST		M		
EST		E		
Fc- γ receptor I A1		M		
Fibrinogen-like 2	+	M		
Galectin 3	+	M		+
GBP1		M		+
GBP2	+	M	+	+
GM2	+	M		
Granzyme A	+	M	+	
Granzyme K		M		
HLA-A	+	E	+	+
HLA-B	+	E		+
HLA-C	+	E	+	
HLA-DMA	+	M	+	
HLA-DMB	+	M		
HLA-DPA1	+	E	+	
HLA-DPB1	+	M	+	
HLA-DQB1	+	E	+	
HLA-DRA	+	E	+	
HLA-DRB3	+	E		
HLA-E	+	E	+	+
IFI16		M		+
IFI27	+	M		
IFI30	+	E		
Isg20	+	E		+
IgH γ 3	+	L		
Ig λ joining 3	+	L	+	
Ig superfamily 6		M		

genes expressed during viral clearance in this animal reflects the amplification and diversification of the intrahepatic inflammatory response and the induction of antiviral activity within the infected cells.

Only two of the clearance-associated genes (i.e., Ig heavy and light chains) reached peak induction late in infection in this animal [Fig. 3 (shown in green) and Table 1], reflecting the expansion of a B cell infiltrate when the viral DNA was disappearing from the liver.

Table 1. (continued)

Gene/EST	Selected as induced during viral clearance	Peak expression* (Ch1627)	Induced during clearance in	
			HCV chimps [†]	HBVtg HCs [‡]
Ig superfamily prot.	+	E		
IgE I receptor		E		
IL10R- α		M	+	
IAN4L1		E		
Karyopherin		M		
LAP3	+	M		+
Leupaxin		M		
Lysosome-assoc.-5	+	E		
Lysozyme	+	M		
MCM6		E		
Palmitoyl thioesterase 1		M		
PI3-kinase		E		
PTTG1		M		
PLAC8		M		+
PRC1		E		
Proteoglycan 1	+	M		
PSMB10 (MECL-1)		M	+	+
PSMB9 (LMP-2)	+	M	+	+
PSME1 (PA28 α)	+	E	+	+
PSME2 (PA28 β)	+	E	+	+
RAB20		M		
RAB27A		M		
RAB31		M		
RAC2	+	M		
RalGDS-like		M		
Regulatory factor X5	+	M		
RARRES3	+	M		
RhoGDI- β		M		
Ribonuclease T2		M		
Ribonuclease reduc. M2		M		
S100A10		E		
Ser/Thr kinase 6		M		
SOD2	+	M		
Solute carrier 7A7	+	M		
SP110		M		
STAT1	+	M	+	+
CHST6		M		
Tapasin	+	E		+
TCR β	+	E		
TCR γ	+	M		
TCR γ locus	+	M		
Thymidylate synthetase		M		
T-LAK prot. kinase		M		
GNMB		M		
TRIM22	+	M	+	
TrpR5	+	M	+	+
TYROBP	+	E	+	
Ubiquitin D	+	M		
UBE2C		M		
UBE2L6	+	M	+	+
Uncoupling prot. 2	+	E		

Table includes genes selected as induced during viral clearance (+) and/or correlated with prototype marker genes as described in the text. chimps, chimpanzees; HCs, hepatocytes; HBVtg, HBV transgenic; reduc., reductase; prot., protein.

*Peak expression early (E), middle (M), or late (L) during viral clearance in Ch1627.

[†]Induced during clearance in hepatitis C virus (HCV)-infected chimpanzees (23).

[‡]Induced by IFN β and/or IFN γ in murine hepatocytes during clearance of HBV (11).

Discussion

The objective of this study was to identify liver genes that are transcriptionally regulated during HBV infection and are associated with either the entry and expansion of the virus or its immune-mediated clearance. The first group could include proviral genes that the virus might induce to optimize replication and antiviral genes that reflect the activation and/or effector functions of the innate immune response. The second group should include genes brought into the liver by cells of the adaptive immune response, plus

Kinetics of Peak Expression Ch 1627

Fig. 3. Kinetics of liver gene expression during viral clearance in Ch1627. Early (blue), Middle (red), and Late (green) genes displayed maximal expression in weeks 12, 14, and 16, respectively.

hepatocellular genes that are activated by those cells and their products, e.g., IFN- γ .

These studies were modeled after a previous report from our laboratory (23) in which the genes associated with HCV spread and clearance were identified in the livers of chimpanzees that were acutely and chronically infected by HCV. Together, the results of the two studies display remarkable differences in the repertoire of genes that are induced during the infection by HBV in this study (no genes) and HCV in previous studies (23, 24) (27 genes, many of which are regulated by IFN- α/β), probably reflecting differences in the genome structure and the replication strategies of the two viruses and corresponding differences in their ability to induce an innate immune response. The results also reveal remarkable similarities in the repertoire of genes induced during viral clearance in both HBV and HCV infections (23, 24) and during T cell-mediated viral clearance in the HBV transgenic mouse model (11) (Table 1), probably reflecting similarities in the adaptive immune mechanisms responsible for their control. The results also illustrate that γ/δ T cells contribute to the inflammatory infiltrate during acute hepatitis B, and they identify a compact subset of candidate antiviral genes, some of which could mediate viral clearance by interrupting the HBV life cycle in infected cells.

IFN- α/β and - γ are known to inhibit viral replication in the liver of HBV transgenic mice (8–10, 25). Both cytokines are produced during the innate immune response, IFN- α/β primarily by infected cells and plasmacytoid dendritic cells (26, 27), and IFN- γ primarily by NK and NKT cells (28), which are abundant in the liver (28, 29). Thus, because we and others have shown that the expression of a large number of IFN-regulated genes is rapidly and consistently induced in the liver of HCV-infected chimpanzees and that the strength of their expression correlates with viremia (23, 24), we expected the same to occur in the HBV-infected animals. Surprisingly, not a single gene was induced or repressed by HBV in all three acutely infected animals. Furthermore, no genes were significantly up- or down-regulated in any of the animals in the lag phase of infection or the log phase of viral spread. These results imply that HBV did not induce an intrahepatic innate immune response that could be detected by gene chip analysis in all of the infected animals. The basis for this is not immediately clear. It may reflect the replication strategy of HBV whose DNA genome replicates within nucleocapsid particles (30) and thus is shielded from the cellular machinery that normally senses double-stranded RNA (31). It also implies that extracellular HBV virions and subviral products are not detected by the toll-like receptor system (32).

Many RNA viruses have developed elegant strategies to evade the antiviral effects of cytokines induced during the innate immune response (26). The need to develop these strategies exists for these

viruses because they induce IFN- α/β in infected cells or IFN- γ by cells of the innate immune system. We suggest that HBV achieves the same end by not inducing these cytokines in the first place. An interesting consequence of this is that by not needing to evade IFN early in the infection, the virus remains susceptible to IFN later in the infection when the adaptive immune response begins (6, 33).

In contrast to the apparent invisibility of HBV to the innate sensing machinery, when CD3⁺ MHC-restricted α/β receptor T cells appeared in the liver of the infected animals [Table 1 and Fig. 2B and 3 (shown in blue)], a temporal and functional cascade was initiated that correlated very strongly with the induction of IFN- γ and viral clearance. Because the IFN- γ -inducible chemokine MIG was induced immediately before the onset of clearance in all three animals (data not shown), it is likely that IFN- γ played a major role in inducing most of the viral clearance-associated genes detected in this study, including 2'5'OAS, which is known to be inducible by IFN- γ (34). By using Ch1627 as a prototype, the early appearance of TCR α/β receptor genes [Table 1 and Fig. 3 (shown in blue)] was accompanied by the induction of IFN- γ -regulated genes (e.g., MHC class I, MHC class II, immunoproteasome components PA28 α and PA28 β , and the TAP-binding protein tapasin), all of which may have facilitated antigen processing and presentation to the incoming T cells. In addition, the early induction of *RANTES*, *MIG*, and *IP-10* probably enhanced the recruitment of additional inflammatory cells into the liver. The remaining early genes underlie a wide array of cellular functions, some of which might contribute to viral clearance. For example, ISG20 is an IFN-inducible RNase that increases the resistance to infection by RNA viruses (35) and is also induced during IFN-mediated suppression of viral replication in HBV transgenic mouse hepatocyte cell lines (11). Thus, ISG20 may also be able to destroy HBV RNA in infected hepatocytes. Additional studies are needed to test this hypothesis.

The early peak was followed by a second group of genes [Table 1 and Fig. 3 (shown in red)] reflecting expansion of the inflammatory infiltrate to include γ/δ T cells. γ/δ T cells do not express CD4 or CD8, do not recognize peptide antigens, and are MHC-unrestricted (36), but they have been shown to produce IFN- γ and contribute to the clearance of other viral infections (36, 37). Thus, further investigation of γ/δ T cell immunobiology in HBV infection appears to be warranted.

The middle gene cluster [Table 1 and Fig. 3 (shown in red)] also displays further evidence of T and NK cells and macrophage activation in the liver, because CD38 and the T cell and NK cell death effectors granzyme A and granzyme K peaked at this time, as did a large number of IFN- γ -regulated genes, consistent with the detection of IFN- γ mRNA and the decreasing viral DNA content of the liver (Fig. 1). For example, *STAT1*, which mediates IFN receptor signaling, was maximally induced at this time, as were several MHC class II genes, the chemokines *IP-10*, *MIG* and *IP-9*, the immunoproteasome components *LMP2* and *MECL-1*, ubiquitin D, and the ubiquitin-conjugating enzymes E2C and E2L6, all of which likely contribute to the inflammatory process. In addition, the IFN- γ -induced GTPases, GBP1 and -2, as well as *IFI27* and *IFI16*, peaked at this time. Interestingly, the murine homologues of *GBP1* and *IFI27* inhibit vesicular stomatitis virus (12) and Sindbis virus infection in mice (38), respectively, and they are induced in IFN-treated murine hepatocyte cell lines when HBV replication is suppressed (Table 1). Thus, further investigation of the role these genes play in viral clearance during HBV infection appears to be warranted.

Last, the middle gene cluster contains several other genes that could play a role in viral clearance and/or disease pathogenesis during HBV infection. Among these are members of the Ras-related family of small GTPases (i.e., *Rab20*, -27a, and -31) (reviewed in ref. 39) that might inhibit HBV replication by virtue of their ability to regulate vesicular transport (39–41). Cathepsin B is a cysteine protease that is released into the cytoplasm (42) during

hepatocyte apoptosis where it might also target HBV. Karyopherin (importin- α) induction might also influence HBV replication because it is known to regulate HBV capsid nuclear import (43).

The last (late) wave consisted of Ig heavy and light chain genes [Table 1 and Fig. 3 (shown in green)], apparently reflecting an influx of B cells into the liver.

Although the results of this study elucidate and strongly underscore the role of the adaptive immune response and its products in viral clearance during acute HBV infection, they do not discriminate among genes that are expressed in the inflammatory cells or the hepatocytes. Although this should be clarified in future studies, some insight can be gained by comparing the current results with our previous analysis of the gene expression profiles of HBV transgenic murine hepatocyte cell lines in which HBV replication was suppressed by IFN- β and - γ *in vitro*. As shown in Table 1, a relatively compact panel of genes [*STAT1*, *IFI16*, *GBP1*, *GBP2*, *LMP2*, *MECL1*, *PA28 α/β* , *IP-10*, *MIG*, *ISG20*, leucine aminopeptidase 3, placenta-specific 8, tryptophanyl-tRNA synthetase (*TrpRS*), β -2-microglobulin, ubiquitin-conjugating enzyme *E2L6* (*UBE2L6*), tapasin, and MHC class I] was induced during viral clearance in all three chimpanzees and in IFN- β - and/or - γ -treated mouse hepatocytes (11), suggesting that they may function as effector molecules that suppress viral replication and contribute noncytolytically to the control of HBV infection. The generality and potential importance of this subset of genes are underscored by the fact that several of them are also induced in the liver during viral clearance in HCV-infected chimpanzees (23, 24).

Conclusion

The current results suggest that HBV acts like a stealth virus early in infection, remaining undetected and spreading until the onset of

the adaptive immune response several weeks later. When MHC-restricted α/β T cells enter the liver and recognize antigen, they kill some of the infected cells and secrete IFN- γ , which induces the expression of a large number of genes that enhance antigen processing and presentation; recruit macrophages, NK cells, and γ/δ T cells that also produce IFN- γ ; and amplify the process. Several of the IFN- γ -induced genes have antiviral activity in other systems, raising the possibility that they may also inhibit HBV replication. These highly coordinated cellular and molecular events continue until the infection is terminated, at which point they rapidly subside. Because many of the same genes are also associated with clearance of HCV, they represent a starting point for further elucidation of the cellular and molecular immunobiology of these infections.

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- Chisari, F. V. & Ferrari, C. (1995) *Annu. Rev. Immunol.* **13**, 29–60.
- Missale, G., Redeker, A., Person, J., Fowler, P., Guilhot, S., Schlicht, H. J., Ferrari, C. & Chisari, F. V. (1993) *J. Exp. Med.* **177**, 751–762.
- Rehermann, B., Fowler, P., Sidney, J., Person, J., Redeker, A., Brown, M., Moss, B., Sette, A. & Chisari, F. V. (1995) *J. Exp. Med.* **181**, 1047–1058.
- Rehermann, B., Chang, K. M., McHutchinson, J., Kokka, R., Houghton, M., Rice, C. M. & Chisari, F. V. (1996) *J. Virol.* **70**, 7092–7102.
- Rehermann, B., Lau, D., Hoofnagle, J. H. & Chisari, F. V. (1996) *J. Clin. Invest.* **97**, 1655–1665.
- Thimme, R., Wieland, S., Steiger, C., Ghayeb, J., Reimann, K. A., Purcell, R. H. & Chisari, F. V. (2003) *J. Virol.* **77**, 68–76.
- Guidotti, L. G., Rochford, R., Chung, J., Shapiro, M., Purcell, R. & Chisari, F. V. (1999) *Science* **284**, 825–829.
- Guidotti, L. G., Ishikawa, T., Hobbs, M. V., Matzke, B., Schreiber, R. & Chisari, F. V. (1996) *Immunity* **4**, 25–36.
- McClary, H., Koch, R., Chisari, F. V. & Guidotti, L. G. (2000) *J. Virol.* **74**, 2255–2264.
- Wieland, S. F., Guidotti, L. G. & Chisari, F. V. (2000) *J. Virol.* **74**, 4165–4173.
- Wieland, S. F., Vega, R. G., Muller, R., Evans, C. F., Hilbush, B., Guidotti, L. G., Sutcliffe, J. G., Schultz, P. G. & Chisari, F. V. (2003) *J. Virol.* **77**, 1227–1236.
- Anderson, S. L., Carton, J. M., Lou, J., Xing, L. & Rubin, B. Y. (1999) *Virology* **256**, 8–14.
- Carlow, D. A., Teh, S. J. & Teh, H. S. (1998) *J. Immunol.* **161**, 2348–2355.
- Guidotti, L. G., Matzke, B., Schaller, H. & Chisari, F. V. (1995) *J. Virol.* **69**, 6158–6169.
- Baugh, L. R., Hill, A. A., Brown, E. L. & Hunter, C. P. (2001) *Nucleic Acids Res.* **29**, E29.
- Schadt, E. E., Li, C., Ellis, B. & Wong, W. H. (2001) *J. Cell. Biochem. Suppl.* **37**, 120–125.
- Li, C. & Hung Wong, W. (2001) *Genome Biol.* **2**, RESEARCH0032.
- Parker, N. & Porter, A. C. (2004) *BMC Genomics* **5**, 8.
- Watts, C. (2001) *Science* **294**, 1294–1295.
- Liu, J., Shue, E., Ewalt, K. L. & Schimmel, P. (2004) *Nucleic Acids Res.* **32**, 719–727.
- Kakimi, K., Lane, T. E., Wieland, S., Asensio, V. C., Campbell, I. L., Chisari, F. V. & Guidotti, L. G. (2001) *J. Exp. Med.* **194**, 1755–1766.
- Losana, G., Bovolenta, C., Rigamonti, L., Borghi, I., Altare, F., Jouanguy, E., Forni, G., Casanova, J. L., Sherry, B., Mengozzi, M., et al. (2002) *J. Leukocyte Biol.* **72**, 735–742.
- Su, A. I., Pezacki, J. P., Wodicka, L., Brideau, A. D., Supkevova, L., Thimme, R., Wieland, S., Bukh, J., Purcell, R. H., Schultz, P. G., et al. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 15669–15674.
- Bigger, C. B., Brasky, K. M. & Lanford, R. E. (2001) *J. Virol.* **75**, 7059–7066.
- Guidotti, L. G., Borrow, P., Hobbs, M. V., Matzke, B., Gresser, I., Oldstone, M. B. & Chisari, F. V. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 4589–4594.
- Katze, M. G., He, Y. & Gale, M., Jr. (2002) *Nat. Rev. Immunol.* **2**, 675–687.
- Siegel, F. P., Kadowaki, N., Shodell, M., Fitzgerald-Bocarsly, P. A., Shah, K., Ho, S., Antonenko, S. & Liu, Y. J. (1999) *Science* **284**, 1835–1837.
- Bendelac, A., Rivera, M. N., Park, S. H. & Roark, J. H. (1997) *Annu. Rev. Immunol.* **15**, 535–562.
- Eberl, G., Lees, R., Smiley, S. T., Taniguchi, M., Grusby, M. J. & MacDonald, H. R. (1999) *J. Immunol.* **162**, 6410–6419.
- Seeger, C. & Mason, W. S. (2000) *Microbiol. Mol. Biol. Rev.* **64**, 51–68.
- Samuel, C. E. (2001) *Clin. Microbiol. Rev.* **14**, 778–809.
- Hertzog, P. J., O'Neill, L. A. & Hamilton, J. A. (2003) *Trends Immunol.* **24**, 534–539.
- Wieland, S. F., Spangenberg, H. C., Thimme, R., Purcell, R. H. & Chisari, F. V. (2004) *Proc. Natl. Acad. Sci. USA* **101**, 2129–2134.
- Guidotti, L. G., Morris, A., Mendez, H., Koch, R., Silverman, R. H., Williams, B. R. & Chisari, F. V. (2002) *J. Virol.* **76**, 2617–2621.
- Espt, L., Degols, G., Gongora, C., Blondel, D., Williams, B. R., Silverman, R. H. & Mecht, N. (2003) *J. Biol. Chem.* **278**, 16151–16158.
- Hayday, A. C. (2000) *Annu. Rev. Immunol.* **18**, 975–1026.
- Wang, T., Scully, E., Yin, Z., Kim, J. H., Wang, S., Yan, J., Mamula, M., Anderson, J. F., Craft, J. & Fikrig, E. (2003) *J. Immunol.* **171**, 2524–2531.
- Labrada, L., Liang, X. H., Zheng, W., Johnston, C. & Levine, B. (2002) *J. Virol.* **76**, 11688–11703.
- Seabra, M. C., Mules, E. H. & Hume, A. N. (2002) *Trends Mol. Med.* **8**, 23–30.
- Trambas, C. M. & Griffiths, G. M. (2003) *Nat. Immunol.* **4**, 399–403.
- Pereira-Leal, J. B. & Seabra, M. C. (2001) *J. Mol. Biol.* **313**, 889–901.
- Guicciardi, M. E., Deussing, J., Miyoshi, H., Bronk, S. F., Svingen, P. A., Peters, C., Kaufmann, S. H. & Gores, G. J. (2000) *J. Clin. Invest.* **106**, 1127–1137.
- Rabe, B., Vlachou, A., Pante, N., Helenius, A. & Kann, M. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 9849–9854.