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Longitudinal Analysis of CD8$^+$ T Cells Specific for Structural and Nonstructural Hepatitis B Virus Proteins in Patients with Chronic Hepatitis B: Implications for Immunotherapy

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The cytotoxic T-cell response in chronic hepatitis B virus (HBV) infection has been described as weak and mono- or oligospecific in comparison to the more robust virus-specific T-cell response present in resolved infection. However, chronic hepatitis B is a heterogeneous disease with markedly variable levels of virus replication and liver disease activity. Here we analyzed (both directly ex vivo and after in vitro stimulation) the HBV-specific CD8 T-cell responses against structural and nonstructural HBV proteins longitudinally in patients with different patterns of chronic infections. We found that the profiles of virus-specific CD8$^+$-T-cell responses during chronic infections are highly heterogeneous and influenced more by the level of HBV replication than by the activity of liver disease. An HBV DNA load of $<10^7$ copies/ml appears to be the threshold below which circulating multispecific HBV-specific CD8$^+$ T cells are consistently detected. Furthermore, CD8$^+$ T cells with different specificities are differentially regulated during chronic infections. HBV core-specific CD8$^+$ T cells are associated with viral control, while CD8$^+$ T cells specific for envelope and polymerase epitopes can occasionally be found in the setting of high levels ($>10^7$ copies) of HBV replication. These findings have implications for the design of immunotherapy for chronic HBV infections.

Hepatitis B virus (HBV) is a hepatotropic, noncytopathic DNA virus which can cause chronic hepatitis, cirrhosis, and liver cancer (19).

The ability to clear HBV after infection has been associated with the presence of a strong virus-specific T-cell response. Multispecific antiviral CD4$^+$- and CD8$^+$-T-cell responses with a type 1 profile of cytokine production are detectable in subjects with self-limited acute hepatitis B (5, 10, 12, 13, 29, 32, 40). Due to the lack of suitable animal models, it has been difficult to establish a causative effect, but recently CD8$^+$-T-cell deletion experiments performed with HBV-infected chimpanzees showed the essential role of HBV-specific CD8$^+$ T cells in HBV control (41).

In contrast to the robust virus-specific CD8$^+$- and CD4$^+$-T-cell responses present in patients with resolved HBV infections, patients with chronic infections are usually characterized by weak virus-specific T-cell responses (2, 10, 12, 13, 28, 40). However, the difference between T-cell responses present in acute or resolved versus chronic hepatitis B has obscured the diversity present within chronic hepatitis B. Chronic hepatitis B is clearly a highly heterogeneous disease, and the levels of virus replication, liver disease activity (19), and humoral responses (23) can differ markedly in patients with chronic hepatitis B. Patients can be characterized by levels of HBV replication ranging from 10^3 to 10^9 HBV DNA copies/ml in the presence or in the absence of liver inflammation.

Furthermore, in some patients, the profile of liver disease and HBV replication is stable, whereas other patients experience episodic flares of disease, with fluctuating levels of HBV DNA. All of these different profiles of disease (in particular, high levels of alanine transaminase [ALT], an enzyme released by lysed hepatocytes) have traditionally been associated with different magnitudes of HBV-specific CD8$^+$-T-cell responses (30), but convincing data demonstrating this association are still lacking. Episodes of acute flares during chronic HBV infection are associated with a recovery of HBV-specific T-helper responses (36, 42), but increased levels of HBV-specific CD8$^+$-T-cell responses have been demonstrated only following the resolution of chronic infection (33), after the inhibition of HBV replication during lamivudine therapy (6, 22), and in patients with a low level of HBV replication and no signs of liver inflammation (20).

Here we carried out a longitudinal study to analyze the dynamic profiles of HBV-specific CD8$^+$-T-cell responses according to the level of virus replication and liver inflammation by using immunological methods (HLA tetramers and intracellular cytokine staining [ICCS]) that allow a precise quantification of virus-specific CD8$^+$ T cells. We also evaluated whether CD8$^+$ T cells specific for different antigenic determinants may differently contribute to viral control. During HBV infection, HBV-specific CD8$^+$ T cells can target different epitopes located within the HBV core (28, 39), envelope (25), polymerase (32), and X (11) proteins. Differences between CD8$^+$ T cells specific for different HBV antigens were recently demonstrated for patients with chronic HBV infection. Envelope-specific CD8$^+$ T cells are not visualized by HLA tetramers (34), and CD8$^+$ T cells specific for structural and nonstruc-

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TABLE 1. Characteristics of patients with chronic HBV infections (HBsAg positive and anti-HBc positive)[a]

Patient	Age (yr)	Sex	Route of infection	Presence (+) or absence (−) of:		HBV genotype	No. of HBV DNA copies/ml	ALT level (U/liter)	Liver histologic feature
				HBeAg	Anti-HBe				
C1	22	F	Neonatal	−	+	D	2.4×10^4	36	ND
C2	35	F	?	−	+	D	2×10^4	28	ND
C3	37	F	?	−	+	ND	1.3×10^4	31	ND
C4	56	F	Sexual (?)	−	+	B	2.7×10^6	74	Min NI, sev fibrosis
C5	61	F	?	−	+	D	1.5×10^6	61	Min NI, sev fibrosis
C6	44	M	Neonatal	−	+	D	1.0×10^6	57	Min NI, min fibrosis
C7	43	M	?	+	−	D	1.0×10^8	227	Mod NI, mod fibrosis
C8	36	M	Neonatal	+	−	A	4.0×10^8	2,635	Mod NI, min fibrosis
C9	25	M	?	+	−	A	5.3×10^8	291	Cirrhosis
C10	49	F	?	+	−	C	5.9×10^8	20	Min NI, min fibrosis
C11	25	M	Neonatal	+	−	A	5.8×10^8	66	Min NI, min fibrosis
C12	32	M	Neonatal	+	−	E	6.8×10^8	60	Mod NI, no fibrosis
C13	25	M	?	+	−	D	10×10^8	54	Mod NI, no fibrosis

[a] F, female; M, male; ?, unknown; ND, not determined; min, minimal; mod, moderate; sev, severe; NI, necroinflammation.

tural HBV antigens are not equally effective in the recognition of HBV-infected hepatocytes in the transgenic mouse model of HBV infection (14). Whether $CD8^+$ T cells specific for different HBV antigens are differentially regulated during chronic hepatitis B, however, has never been evaluated.

MATERIALS AND METHODS

Patients. A total of 25 HLA-A2-positive patients with HBV infection were selected for this study. Screening for HLA-A2 positivity was performed by staining peripheral blood mononuclear cells (PBMC) from patients with a fluorescent anti-HLA-A2.01 antibody (Serotec); selected patients were confirmed to have the HLA-A2.01 allele by PCR DNA typing. A total of 12 patients had clinical, biochemical, and virological evidence of resolved acute HBV infection (recovered from acute hepatitis B between 6 months and 3 years before, normal ALT levels, positive for antibody to the HBV core [anti-HBc], and negative for HBV surface antigen [HBsAg]); the remaining 13 patients had clinical, biochemical, and virological evidence of chronic HBV infection (HBsAg positive, anti-HBc positive, and negative for antibodies to hepatitis C virus, delta virus, human immunodeficiency virus type 1 [HIV-1], and HIV-2) (Table 1). Patients C5 and C12 had completed a course of antiviral therapy 9 and 13 months before entering the study, with no benefit, but no other patients had been treated. These 13 patients also had no other causes for chronic liver damage, such as alcohol use, drug use, congestive heart failure, or autoimmune diseases. A longitudinal analysis of antiviral T-cell immunity in the periphery, with a cross-sectional analysis of responses within the liver, was approved by the local ethics committee, and all patients provided written informed consent.

Virological assessments. HBsAg, antibody to the HBV surface, total and immunoglobulin M anti-HBc, HBV envelope antigen (HBeAg), antibody to the HBV envelope (anti-HBe), and antibodies to delta virus, hepatitis C virus, HIV-1, and HIV-2 were determined by using commercial enzyme immunoassay kits (Abbott Laboratories, North Chicago, Ill.; Ortho Diagnostics, Raritan, N.J.; and Sanofi Diagnostic Pasteur, Marnes la Coquette, France). Serum HBV DNA was quantified by using an Amplicor Monitor assay (Roche Pharmaceuticals Ltd., Branchburg, N.J.) with a DNA detection limit of 400 copies/ml (0.0014 pg/ml).

PCR and HBV DNA sequencing. DNA was extracted from serum samples taken at the time of liver biopsy by using a QIAamp DNA blood minikit (Qiagen, Crawley, United Kingdom). HBV DNA was amplified with primers specific for the HBV core and envelope genes as described previously (24). The amplicons were purified, and the core or envelope regions were sequenced directly by using an ABI 377 automated sequencer (Applied Biosystems). The HBV genotype of the predominant virus population in chronically infected patients was determined by analyses of sequenced portions of the core and surface antigens (26).

Synthetic peptides. Peptides corresponding to various HBV-specific HLA-A2-restricted CD8 epitopes were purchased from Chiron Mimotopes (Clayton, Victoria, Australia) or from Primm (Milano, Italy). The purity of the peptides was determined to be greater than 90% by high-pressure liquid chromatography analysis. The peptides were based on HBV genotype D (serotype ayw); the degree of homology among the various genotypes is shown in Table 2.

Isolation of PBMC and production of T-cell lines. PBMC were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation and suspended at concentrations of 1.5×10^6 to 2×10^6/ml in RPMI 1640 (Gibco)–10% fetal calf serum (FCS). Cells were stimulated with 1 μM concentrations of various peptides in a 96-well plate. Recombinant interleukin 2 (R&D Systems, Abingdon, United Kingdom) was added on day 4 of culturing, and cells were analyzed after 10 to 12 days of culturing. PBMC stimulation was performed with a panel of 11 peptides (Table 2) corresponding to HBV sequences previously identified as HLA-A2-restricted cytotoxic T-lymphocyte epitopes (4).

Purification of T cells from liver biopsy specimens. Mononuclear cells were purified from biopsy specimens. Liver tissue not needed for diagnostic purposes was extensively washed in RPMI 1640 and then digested with collagenase (1 mg/ml; Sigma Chemical Co., St. Louis, Mo.) and DNase (25 μg/ml; Sigma Chemical Co.) for 1 h at 37°C. The cell suspension was washed twice, and mononuclear cells were recovered by centrifugation over a Ficoll-Hypaque density gradient.

Intracellular IFN-γ production. For analysis of gamma interferon (IFN-γ) production, ex vivo-purified PBMC or short-term T-cell lines were stimulated at 2×10^6 to 3×10^6/ml in RPMI 1640–10% FCS with HBV peptides (1 μM) or with HepG2 cell lines ($HLA\text{-}A2^+$) supporting full HBV replication (37) for 6 h at 37°C in the presence of brefeldin A (10 μg/ml; Sigma-Aldrich, Poole, Dorset, United Kingdom). Cells were washed, stained with Cy-chrome-conjugated anti-CD8 antibodies (Sigma Chemical Co.), permeabilized, and fixed with Cytofix/Cytoperm (Pharmingen, San Diego, Calif.) according to the manufacturer's instructions. Fluorescein isothiocyanate-conjugated anticytokine antibodies or isotype-matched control antibodies were added (30 min, 22°C), and the cells were washed twice and analyzed by flow cytometry.

Staining with HLA tetramer complexes. HLA class I tetramers were produced as described previously (21) and lately purchased commercially (Proimmune, Oxford, United Kingdom). Directly purified circulating cells or T-cell lines or T cells purified from liver biopsy specimens were incubated for 30 min at 37°C with 1 μg of phycoerythrin-labeled tetramer complex in RPMI 1640–10% FCS in round-bottom polystyrene tubes (Becton Dickinson, Paramus, N.J.). Cells were washed in phosphate-buffered saline and incubated at 4°C for 30 min with saturating concentrations of Cy-chrome-conjugated anti-CD8 antibodies. After further washings, cells were analyzed by using FACSort (Becton Dickinson) and CELLQuest software immediately or after the addition of 1% paraformaldehyde.

RESULTS

Longitudinal analysis of HBV-specific $CD8^+$ T cells in patients with chronic hepatitis B. Thirteen patients with chronic hepatitis B were studied longitudinally for at least 6 months. The clinical and virological features of the patients at the time

TABLE 2. Peptides used for analysis of HBV-specific CD8+-T-cell responses

Peptide	Sequence	Change in epitope sequence in the following HBV genotype[a]:						HLA-A2.01 binding affinity[b] (nM)
		A	B	C	D	E	F	
Core 18–27	FLPSDFFPSV	=	I27[c]	I27	=	=	I27	3.3
Core 107–115	CLTFGRETV	=	=	=	=	=	=	ND
Pol 455–463	GLSRYVARL	=	=	=	=	=	=	71
Pol 502–510	KLHLYSHPI	=	=	=	=	=	=	17
Pol 575–583	FLLSLGIHL	=	=	=	=	=	V579	10
Pol 655–663	ALMPLYACI	=	=	=	=	T661	N661	10
Pol 816–824	SLYADSPSV	=	=	=	=	V820	=	14
Env 183–191	FLLTRILTI	=	K185	=	=	K185	K185	7
Env 335–343	WLSLLVPFV	=	=	A340	=	=	Q341	7
Env 338–347	LLVPFVQWFV	=	=	A340	=	A347	Q341	3.2
Env 348–357	GLSPTVWLSV	A357	=	=	=	=	L356	19

[a] NCBI accession numbers of the HBV genotypes used to analyze amino acid variability in the HBV epitopes were as follows: genotype A, X51970; genotype B, M54923; genotype C, D00630; genotype D, J02203; genotype E, X75664; and genotype F, X75658. =, the sequence of the HBV epitope was identical to that of the peptide.

[b] Binding is expressed as the dose of the tested peptide yielding 50% inhibition of radiolabeled peptide binding to HLA-A2 molecules. The data reported are from the study of Bertoni et al. (4). ND, not determined.

[c] The NCBI accession number for HBV genotype B with isoleucine at position 27 (I27) is the same as that listed in footnote *a*. The NCBI accession number for HBV genotype B with valine instead of isoleucine at position 27 is D00329.

of selection are shown in Table 1. Patients were initially selected for their levels of ALT and HBV DNA, HBeAg status (presence or absence of HBeAg or anti-HBe), and presence or absence of hepatitis flares. We divided them into four categories: (i) group A—patients C1, C2, and C3, anti-HBe positive and stable levels of ALT (<40 U/liter) and HBV DNA ($<10^6$ copies/ml); (ii) group B—patients C4, C5, and C6, anti-HBe positive, ALT level of >40 U/liter, and HBV DNA level of $<10^7$ copies/ml; (iii) group C—patients C7, C8, and C9, HBeAg positive, anti-HBe negative, ALT level of >100 U/liter, HBV DNA level fluctuating from 10^3 to $>10^7$ copies/ml, and episodes of hepatic flares; and (iv) group D—patients C10, C11, C12, and C13, HBeAg positive, anti-HBe negative, ALT level of <70 U/liter, HBV DNA level of $>10^7$ copies/ml, and stable profile of disease. Two patients who recovered from acute hepatitis B were also monitored longitudinally for 3 years after recovery.

Figure 1 shows the complete set of HBV-specific CD8+-T-cell analyses performed for a representative patient from each chronic HBV infection group (Fig. 1a and b) and for one patient who recovered from acute hepatitis (Fig. 1c). The frequency of HBV-specific CD8+ T cells was calculated directly ex vivo with HLA-A2 tetramers (specific for core positions 18 to 27 [core 18–27], envelope positions 183 to 191 [env 183–191] and 348 to 357 [env 348–357], and polymerase positions 816 to 824 [pol 816–824]) (20) or by ICCS with the following peptides: core 18–27, env 183–191, and pol 455–463 (polymerase positions 455 to 463). The presence of HBV-specific CD8+ cells was also analyzed after in vitro expansion of PBMC stimulated with a larger set of HBV peptides corresponding to known HLA-A2-restricted epitopes (Table 2). The short-term lines produced after in vitro stimulation of PBMC with individual peptides were tested for the presence of peptide-specific IFN-γ-producing cells by ICCS. The frequency of HBV-specific CD8+ T cells was quantified by calculating the frequency of IFN-γ CD8+ T cells restimulated with the stimulatory peptides minus the frequency of IFN-γ CD8+ T cells stimulated with an irrelevant peptide or with no peptide.

Direct ex vivo frequency of circulating HBV-specific CD8+ T cells. Results of the analyses of the direct ex vivo frequency of HBV-specific CD8+ T cells with HLA-A2 tetramers and by ICCS for 13 HLA-A2-positive chronically infected patients and 2 patients who recovered from acute HBV infection are summarized in Fig. 2. Differences in the profiles of virus-specific CD8+ T cells were found among the patients in the chronic hepatitis B groups.

HBV-specific CD8+ T cells were not detected directly ex vivo in patients with stable high levels of HBV DNA replication (group D; HBV DNA level of $>10^7$ copies/ml, HBeAg positive) and either normal ALT levels (<35 U/liter) or elevated ALT levels (~70 U/liter). Four patients were analyzed at 11 different times (a total of 59 different tests), but HBV-specific CD8+ T cells were not visualized either with HLA-tetramers or by ICCS. In contrast, HBV-specific CD8+ T cells could be visualized directly ex vivo in other groups of chronic hepatitis B patients characterized by lower levels of HBV replication.

CD8+ cells specific for core 18–27 (core 18–27-specific CD8+ cells) could be visualized only in patients with levels of HBV replication of $<10^7$ copies/ml either with tetramers or by ICCS. Frequencies were low (never higher than 0.15% with the core 18–27 tetramer) but occasionally were comparable to those found in patients with resolved hepatitis (Fig. 2).

We also detected a high frequency of core 18–27-specific CD8+ cells in one chronically infected patient during an episode of a hepatic flare (Fig. 1b, patient C7). This patient was characterized by fluctuating levels of HBV DNA (from 8×10^4 to 4×10^8 copies/ml), and the high frequency of core 18–27-specific CD8+ T cells was visualized with tetramers only at the time of the flare characterized by levels of HBV DNA of $<10^7$ copies/ml, which preceded HBeAg seroconversion. Note that these core 18–27-specific CD8+ T cells were unable to produce IFN-γ or to expand (Fig. 1a, patient C7). Importantly, the other two hepatic flares present in patient C7 (Fig. 1b), as well as two other distinct flares present in patients C8 and C9 (data

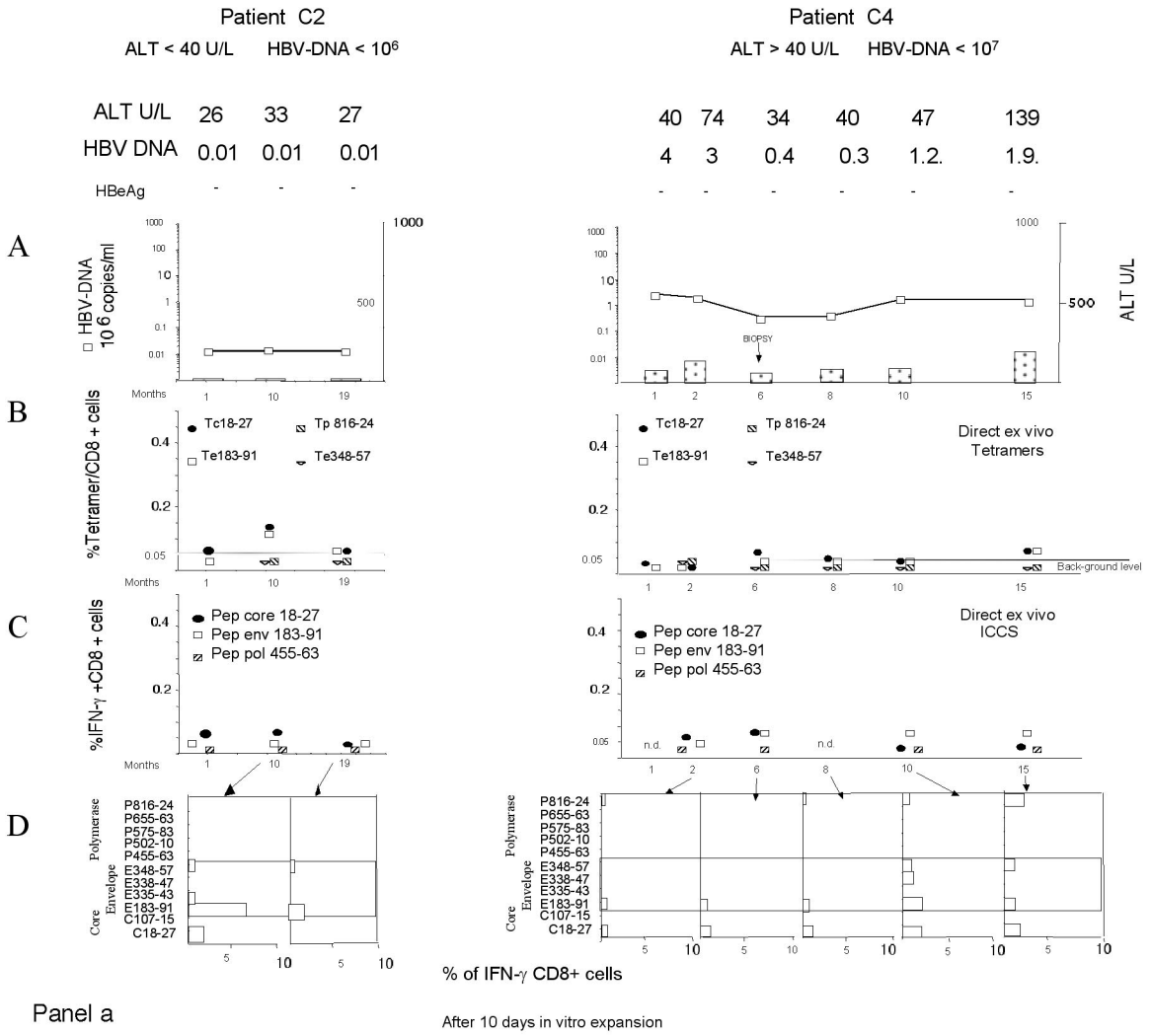

FIG. 1. Longitudinal analysis of HBV-specific $CD8^+$-T-cell responses. Patients with different clinical profiles of HBV infection were studied longitudinally for ALT and HBV DNA levels and the frequencies of HBV-specific $CD8^+$ T cells directly ex vivo and after in vitro expansion. A representative patient for each clinical group is shown: chronically infected patients C2, C4, C7, and C10 in panels a and b and patient R1, with resolved acute hepatitis B, in panel c. (A) Time course analysis of HBV DNA and ALT levels. Serum ALT levels (units per liter [U/L]) (bars) and HBV DNA levels (copies per milliliter) (squares) were analyzed longitudinally. Time zero in patient R1 is the day of clinical onset. n.d., not determined. (B) Percentages of $CD8^+$ T cells that were tetramer positive at each time point. PBMC were tested directly ex vivo with the indicated tetramers (Tc18-27, core 18–27 tetramer; Te183-91, env 183–191 tetramer; Tp816-24, pol 816–824 tetramer; Te348-57, env 348–357 tetramer). The background level of direct ex vivo tetramer staining (0.05%, indicated by a horizontal line) was calculated in HLA-A2-positive, noninfected subjects and in HLA-A2-negative, HBV-infected patients (21). (C) Percentages of peptide-specific IFN-γ-producing $CD8^+$ T cells determined directly ex vivo at different time points. PBMC were stimulated directly ex vivo with the indicated peptides (Pep). The percentages of IFN-γ-producing $CD8^+$ T cells were calculated after subtraction of doubly positive cells obtained from nonstimulated PBMC. (D) Percentages of peptide-specific IFN-γ-producing $CD8^+$ T cells after in vitro expansion (bars). PBMC were stimulated with the indicated peptides. After 10 days of in vitro expansion, the percentages of peptide-specific IFN-γ-producing $CD8^+$ T cells were calculated.

not shown), were not coupled with an increase in the frequency of HBV-specific $CD8^+$ T cells.

Envelope-specific $CD8^+$ cells could be detected directly ex vivo in four out of six chronically infected patients with levels of HBV DNA of $<10^7$ copies/ml but only in two out of seven patients with levels of HBV DNA of $>10^7$ copies/ml (patient C7, env 183–191-specific $CD8^+$ cells; and patient C8, env 348–357-specific $CD8^+$ cells) (data not shown). However, envelope-specific $CD8^+$ cells could be detected at a very low frequency (often $<0.05\%$) only by ICCS and not with the specific HLA-A2 tetramer (Fig. 2). A characterization of tetramer-negative $CD8^+$ cells specific for env 183–191 and env 348–357 epitopes was recently reported in a cross-sectional study (34). Here we were able to monitor the frequencies of env 183–191-specific $CD8^+$ T cells for more than 1 year in at least three patients (patients C4, C5, and C7). The frequencies were remarkably stable over time (Fig. 1, patients C4 and C7). The results obtained for patient C7 (Fig. 1a), who displayed episodes of acute disease flares, were particularly interesting. Despite fluctuating levels of HBV DNA, the absence of mutations

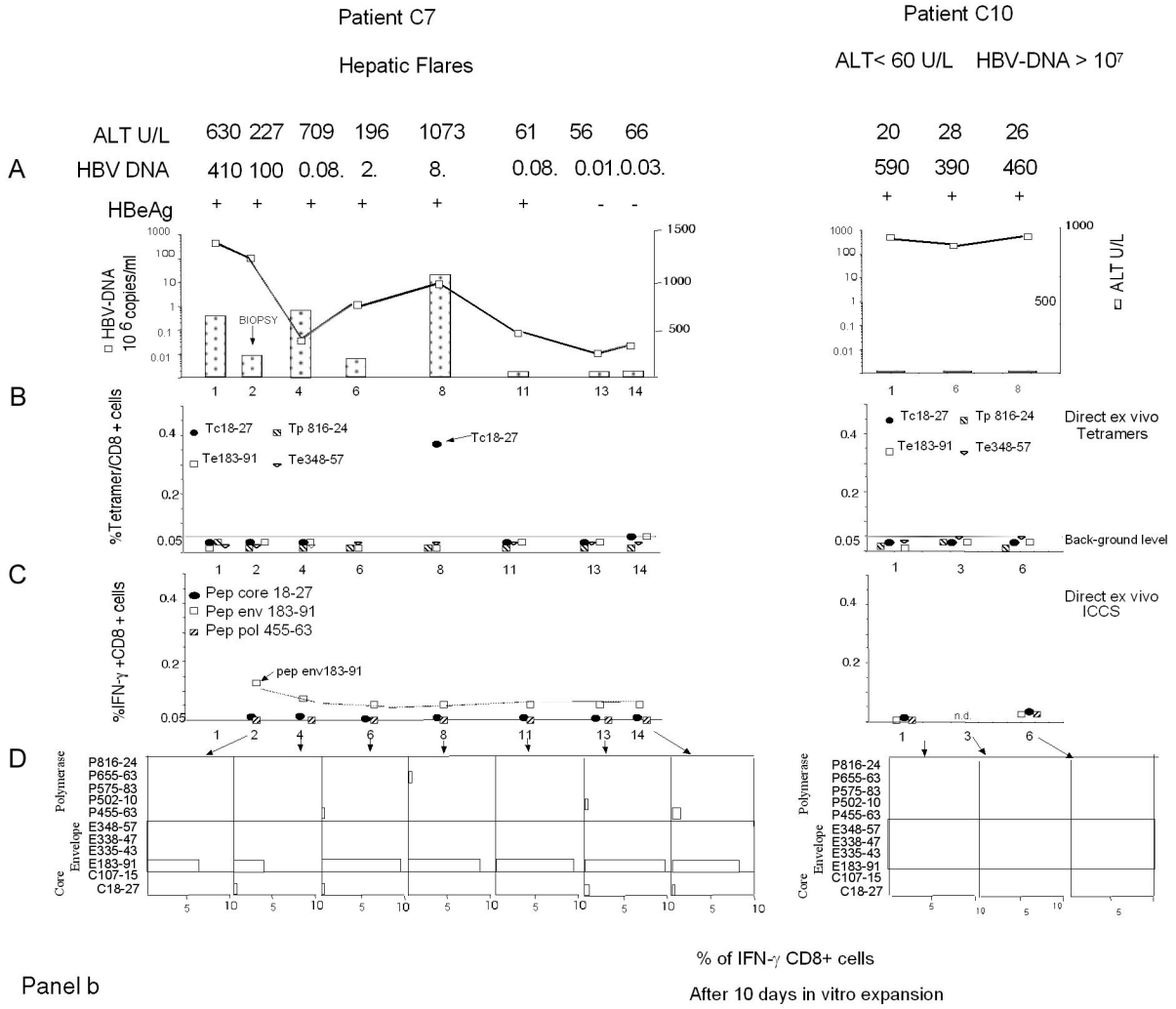

FIG. 1—*Continued*.

in the relevant env 183–191 epitope (Table 3), and ALT levels falling from 1,073 to 60 U/liter, the direct ex vivo frequency (and the capacity for in vitro expansion) of env 183–191-specific $CD8^+$ T cells remained unchanged. This stability contrasts with the behavior of core 18–27-specific $CD8^+$ cells present in the same patients and with that observed during acute hepatitis, where numbers of circulating CD8 T cells increased in proportion to liver enzyme levels and decreased sharply after recovery (21).

Frequency of HBV-specific $CD8^+$ T cells after in vitro expansion. The different sensitivities of direct ex vivo analysis and analysis after in vitro expansion for HBV-specific $CD8^+$-T-cell responses are shown in Fig. 3. For a representative subject (R1), HBV-specific $CD8^+$ T cells were demonstrated after a round of in vitro expansion but would have been missed by direct ex vivo analysis. The profiles of HBV-specific $CD8^+$ T cells after in vitro expansion in all patients are shown in Fig. 4. The results obtained by analysis after in vitro expansion confirmed the patterns observed after direct ex vivo analysis. In the absence of mutations in the epitope (Table 3), circulating core 18–27-specific $CD8^+$ T cells were detectable only when HBV DNA levels were $<10^7$ copies/ml (Fig. 1 and 4, patients C1, C4, and C7). In contrast, efficient expansion of envelope- and polymerase-specific $CD8^+$ T cells was seen even with HBV DNA levels of $>10^7$ copies/ml (Fig. 1 and 4, patient C7).

No correlation between the level of ALT and the detection of HBV-specific $CD8^+$ T cells was found.

The different relationships of the core-, envelope-, and polymerase-specific $CD8^+$ T cells to the level of HBV DNA replication are evident in Fig. 5. The frequency of epitope-specific $CD8^+$ cells after in vitro expansion at all times was plotted against the level of HBV DNA present at the time of the analysis for the various patients. The in vitro expansion of circulating core 18–27-specific $CD8^+$ T cells was inversely proportional to the quantity of HBV DNA. A large expansion of IFN-γ-producing core 18–27-specific $CD8^+$ T cells was present in patients with resolved acute hepatitis (HBV DNA undetectable), a lower level of expansion was present in patients with HBV DNA levels of $<10^7$ copies/ml, and no expansion was present in patients with HBV DNA levels of $>10^7$ copies/ml. In contrast, the expansion of pol 455–463-, pol 816–824-, env

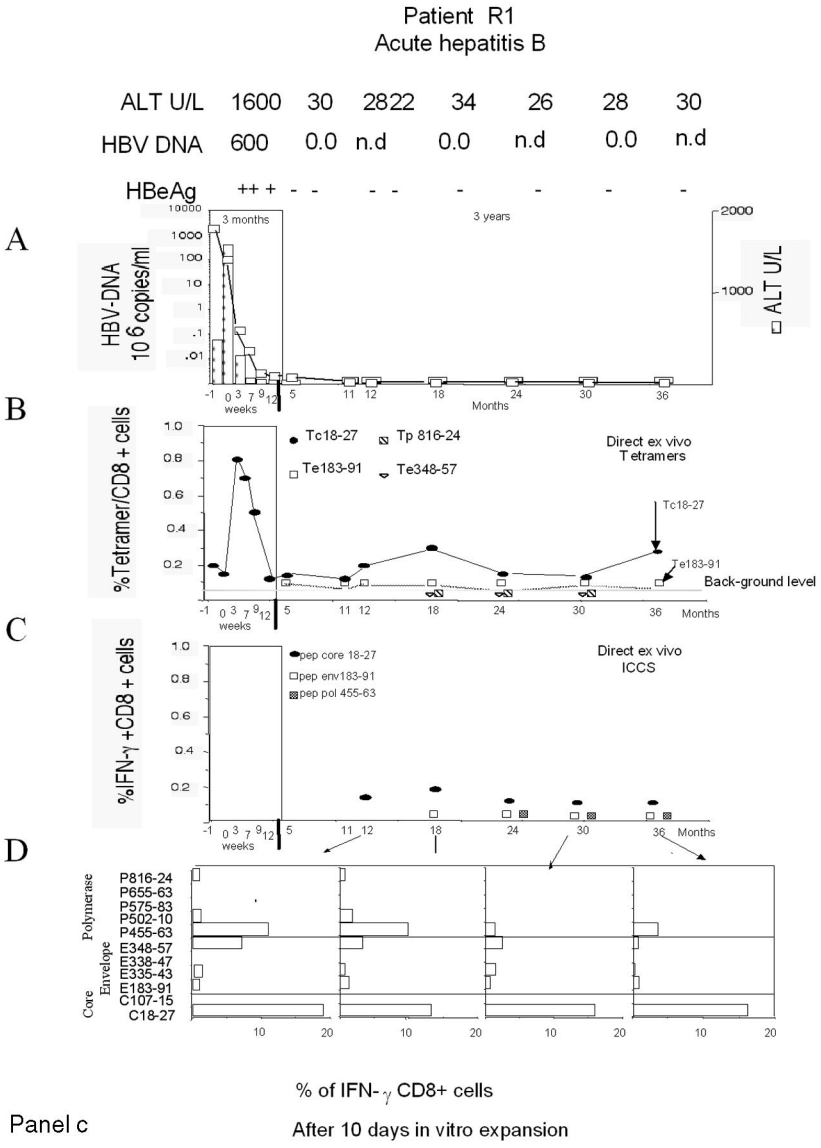

FIG. 1—*Continued*.

183–191-, and env 348–357-specific $CD8^+$ T lymphocytes was less influenced by HBV DNA replication and was also seen in some patients with HBV DNA levels of $>10^7$ copies/ml and without amino acid mutations in the viral epitope (Table 3).

Patients with chronic HBV infections and with HBV DNA levels of $<10^7$ copies/ml demonstrate multispecific $CD8^+$-T-cell responses. The analysis of HBV-specific $CD8^+$ T cells after a round of in vitro expansion with the 11 different HBV peptide epitopes showed that several chronically infected patients possessed $CD8^+$ T cells specific for multiple HBV determinants (Fig. 4). We compared the extents of multispecificity present in patients with resolved infections and persistently infected patients. PBMC from 12 HLA-A2-positive patients who had recovered from acute HBV infections were stimulated with the 11 different peptides used in the longitudinal study. Figure 6 shows that the numbers of HBV epitopes that could be recognized by cells from patients with resolved acute and chronic HBV infections were similar. In particular, cells from all three chronically infected patients with HBV DNA levels of $<10^7$ copies/ml and signs of inflammatory liver disease could recognize at least 4 of the 11 HBV epitopes tested. This degree of multispecificity was higher than or identical to that seen in 6 of 12 patients with resolved acute HBV infections.

Influence of intrahepatic sequestration and of epitope mutations on core 18–27-specific $CD8^+$ T cells. The failure to detect circulating core 18–27-specific $CD8^+$ T cells in patients with HBV DNA levels of $>10^7$ copies/ml might be explained by intrahepatic localization of these cells. To address this possibility, we analyzed the direct ex vivo frequency of core 18–27-specific $CD8^+$ cells in the liver of patients with chronic hepatitis B. The frequency of intrahepatic T cells found by HLA tetramer staining in relation to the HBV DNA levels at the time of liver biopsy clearly showed that core 18–27-specific

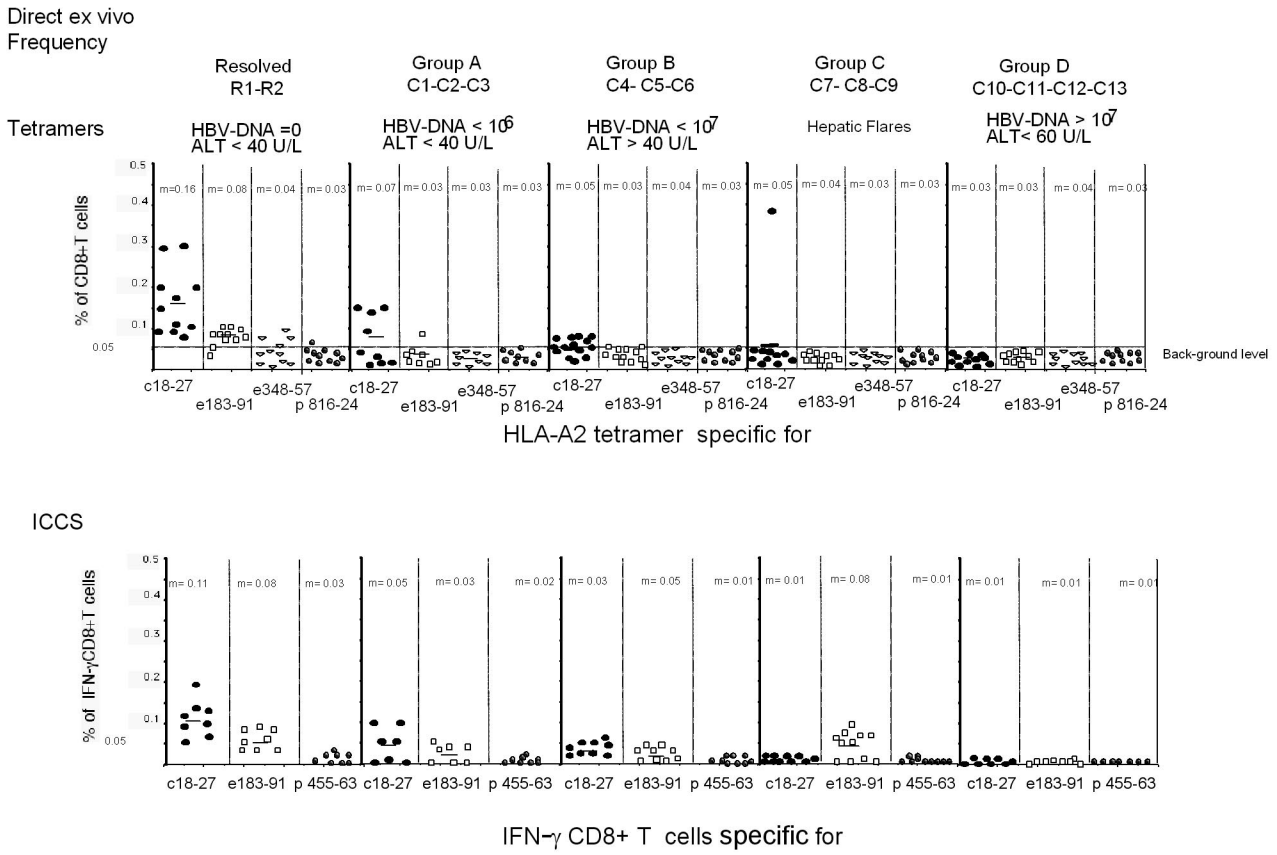

FIG. 2. Direct ex vivo frequencies of HBV-specific CD8+ T cells. The direct ex vivo frequencies of CD8+ T cells stained with HLA tetramers (Tc18–27, Te183–191, Te348–357, and Tp816–824) or producing IFN-γ after stimulation with core 18–27, env 183–191, and pol 455–463 peptides are shown for the indicated groups of patients. The frequency of IFN-γ-producing CD8+ T cells stimulated by the single peptides was subtracted from the frequency obtained in non-peptide-stimulated PBMC. All of the data obtained at different time points for each patient are shown. m, mean. HBV DNA levels are given in copies per milliliter; ALT levels are given in units per liter (U/L).

CD8 +T cells do not accumulate in the liver in proportion to HBV DNA levels (Fig. 7). On the contrary, and in keeping with previous results (20), the frequency of core 18–27-specific CD8+ T cells was inversely proportional to the level of HBV replication (P value, <0.0001, as determined by the Spearman correlation test), and these cells were not found in the liver of patients with serum HBV DNA levels of $>10^7$ copies/ml. The only exception was patient C7, who had core 18–27-specific CD8+ T cells at a frequency of 0.3% of total intrahepatic CD8 T cells. Thus, the absence of circulating core 18–27-specific CD8+ T cells in patients with a high level of HBV replication cannot be explained by preferential intrahepatic compartmentalization.

TABLE 3. Viral mutations in selected HLA-A2-restricted epitopes

Patient	CD8+-T-cell response of and mutation in the following peptide (sequence)[a]:			
	Core 18–27 (FLPSDFFPSV)	Env 183–191 (FLLTRILTI)	Env 348–357 (GLSPTVWLSV)	Pol 455–463 (GLSRYVARL)
C2	+	+	+	− (ND)
C4	+ (I to V 27)	+	+	−
C5	+	+	+	−
C6	+	+	− (V to A 352)	+
C7	+	+	−	−
C8	−	−	− (A to V 357)	+
C9	−	−	− (A to V 357)	+
C10	− (V to I 27)	−	−	− (P to S 457)
C11	−	−	− (ND)	−
C12	−	+	− (ND)	−
C13	−	−	− (ND)	− (ND)

[a] Presence (+) or absence (−) of an epitope-specific CD8+-T-cell response. Mutations are shown in parentheses; e.g., I to V 27 represents a mutation from isoleucine to valine at position 27. ND, not determined.

The absence of core 18–27-specific CD8+ T cells is also not caused by viral mutations resulting in specific epitope inactivation (27). The core protein of HBV strains involved in chronic infections was sequenced. Except for patient C10, infected with an HBV strain carrying an isoleucine-to-valine mutation at position 27, viral mutations could not be found within the core 18–27 epitope or within flanking residues in patients with HBV DNA levels of $>10^7$ copies/ml (Table 3). Note that this isoleucine-to-valine mutation is not associated with core 18–27 unresponsiveness, since this amino acid mutation was also detected in patient C4, who showed a CD8+-T-cell response against this epitope (Table 3).

Functional avidity of HBV-specific CD8+ T cells present in patients with resolved or chronic HBV infections. The persistence of virus-specific CD8 T cells during chronic infection can depend on the avidity of T cells for their target or on the different quantities of virus antigens displayed by infected cells.

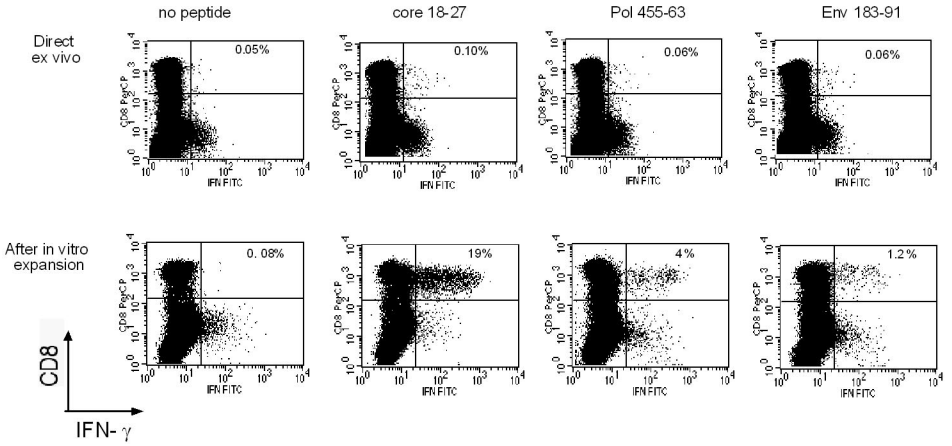

FIG. 3. Analysis of IFN-γ-producing $CD8^+$ T cells directly ex vivo or after in vitro expansion. IFN-γ-producing $CD8^+$ T cells were tested directly ex vivo in PBMC (upper dot plots) or after 10 days of in vitro expansion with the corresponding peptides (lower dot plots). The results shown are from experiments performed with PBMC from patient R1. PBMC were stimulated with the indicated individual peptides either for direct ex vivo analysis or for experiments performed after 10 days of in vitro expansion. In the experiments performed after in vitro expansion, the growing cells were restimulated either with no peptide or with the initial stimulatory HBV peptide. Subdominant peptides (pol 455–463 and env 183–191) tested positive only after 10 days of in vitro expansion.

High-affinity T cells are more likely to be deleted than low-affinity ones, and viral epitopes that are displayed at higher levels appear to drive faster T-cell deletion (1, 24). We investigated whether chronic HBV infection could have selected HBV-specific $CD8^+$ T cells with a low avidity. The activation of core 18–27- and env 183–191-specific $CD8^+$-T-cell lines from both patients with resolved HBV infections and patients with chronic HBV infections was analyzed by measuring IFN-γ production induced by target cells pulsed with decreasing peptide concentrations. T-cell lines were generated from patients with different levels of HBV DNA. Although T-cell lines from patients with resolved infections had a higher frequency of core- or envelope-specific $CD8^+$ T cells, we did not find any evidence of selection of low-avidity HBV-specific T cells in chronically infected patients. Core 18–27-specific $CD8^+$ T cells produced in both patients with chronic infections and patients with resolved infections efficiently recognized target cells pulsed with 10^{-3} μM peptide. There was also no detectable difference in T-cell avidity between env 183–191-specific $CD8^+$ T cells from patients with resolved infections and those from patients with chronic infections, both of which recognized target cells pulsed with 10^{-2} μM peptide (Fig. 8). Core- and envelope-specific $CD8^+$ cell lines from patients with chronic infections and patients with resolved infections were both able to recognize HepG2 cells transfected with the entire HBV genome (37) (data not shown).

DISCUSSION

The HBV-specific $CD8^+$-T-cell response in patients with chronic HBV infections has been studied to date by using cytotoxic experiments performed after several rounds of in vitro expansion (2, 28, 31, 32, 38). Few studies have attempted to evaluate this response directly ex vivo (12, 20, 40). Here we used a combination of methods (HLA tetramers and ICCS) directly ex vivo and after in vitro expansion to increase our ability to study virus-specific $CD8^+$ T cells. Due to the functional alterations of $CD8^+$ T cells caused by chronic viral infection (34), immunological studies performed with single detection methods might underestimate the quantity of virus-specific $CD8^+$ T cells.

The results obtained here confirm that the HBV-specific CD8 T-cell response is weak in the blood of patients with chronic HBV infections (9), with the direct ex vivo frequency of HBV-specific cells usually being <0.1 to 0.2% of total $CD8^+$ T cells. Despite this low frequency, the majority of patients with chronic hepatitis B possess HBV-specific $CD8^+$ T cells, in line with the results of previous investigations of the helper T-cell response (18, 23). Circulating HBV-specific $CD8^+$ T cells were demonstrated in 10 out of 13 patients with chronic hepatitis B (76%), even though analysis of the $CD8^+$-T-cell response was measured with a selected repertoire of HLA-A2-restricted epitopes which is not fully representative of the overall CD8 response (16).

An important finding of this study is that we did not observe a correlation between the profile of circulating HBV-specific $CD8^+$ T cells and the degree of liver damage. Higher frequencies of HBV-specific $CD8^+$ T cells were not present in patients with higher levels of transaminases (Fig. 2). This lack of correlation between HBV-specific $CD8^+$ T cells and liver damage was also supported by results obtained during episodes of acute clinical flares (i.e., ALT levels of >400 U/liter). In four out of five flares, studied in three different patients, we were unable to detect any increase in the frequency of circulating HBV-specific $CD8^+$ T cells. Only in a single case, in which the flare preceded HBeAg seroconversion and occurred with HBV DNA levels of $<10^7$ copies/ml, were we able to detect a sharp increase in the frequency of core 18–27-specific $CD8^+$ T cells. It is possible that our inability to correlate $CD8^+$-T-cell frequency with the extent of liver damage results from the fact that events in the periphery poorly reflect those occurring within the liver. We cannot exclude the possibility that recovery of the HBV-specific T-cell response takes place exclusively within the liver during flares. Furthermore, $CD8^+$-T-cell

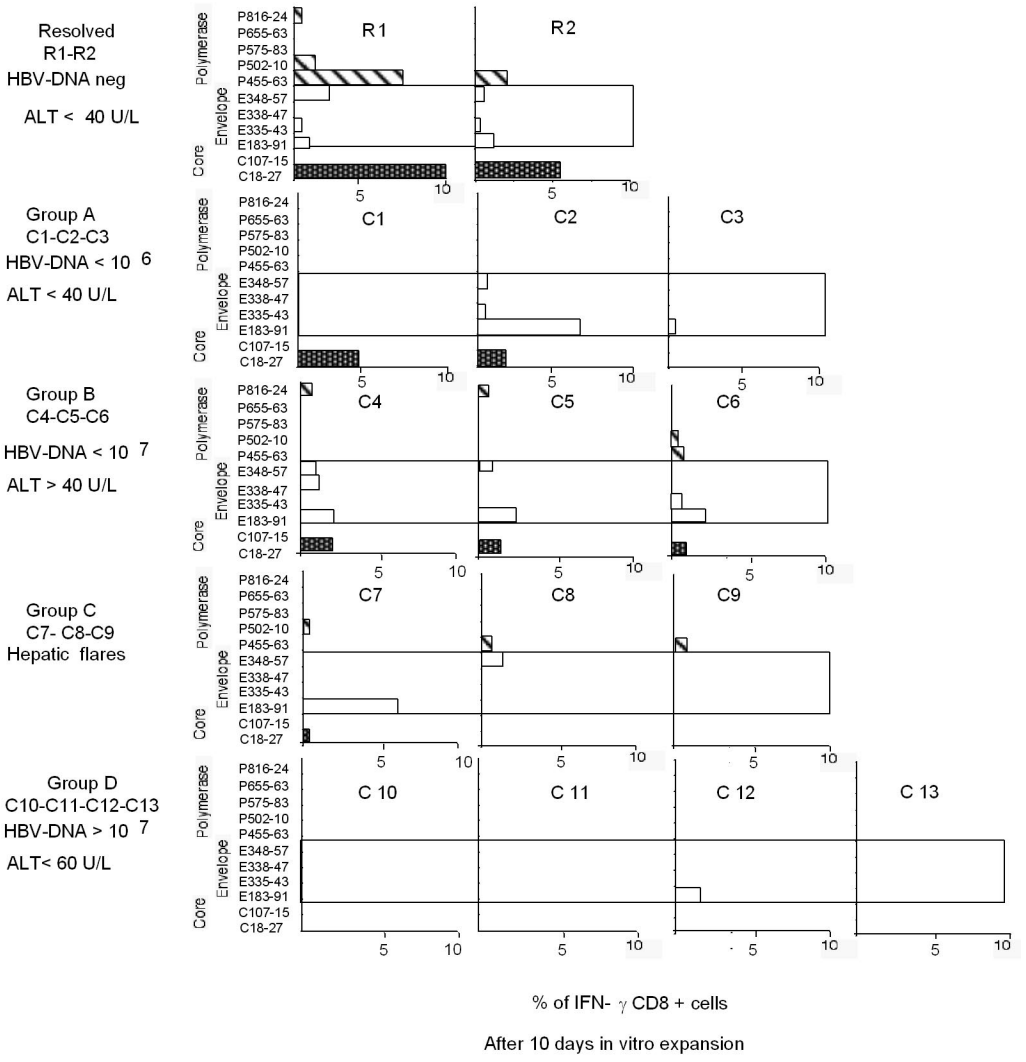

FIG. 4. Profiles of HBV-specific CD8+ responses obtained after in vitro expansion in various patients. Peptide-specific IFN-γ-producing CD8+ T cells were quantified after 10 days of in vitro expansion with the indicated peptides. One representative experiment for each patient is shown. Results for patients R1 and R2 were obtained, respectively, at 12 (R1) and 18 (R2) months after the resolution of acute hepatitis. Results for patients C2, C4, C7, and C10 represent the results shown in Fig. 1a and b at month 10 (C2 and C4), month 8 (C7), and month 3 (C10). HBV DNA levels are given in copies per milliliter; ALT levels are given in units per liter (U/L).

epitopes other than those analyzed in this study might play a role in the pathogenesis of hepatic flares. Nevertheless, we did not find any consistent association between the HBV-specific CD8+-T-cell response and the extent of liver damage, in contrast to the clear association between the presence of these cells and viral load. We consistently detected a higher frequency of HBV-specific CD8+ T cells in patients with a low level of HBV replication than in those with a high level of HBV replication. This finding is in agreement with the results of previous studies carried out with fewer cytotoxic T-lymphocyte epitopes (20, 40) and in those performed after spontaneous (33) or treatment-induced (6, 33) reduction of HBV replication.

A serum HBV DNA concentration of about 10^7 copies/ml appears to be the dividing threshold for consistent detection of circulating HBV-specific CD8+ T cells. Below this concentration, HBV-specific CD8+ T cells could be detected both directly ex vivo and after in vitro expansion; in addition, the CD8 response was often multispecific, with CD8+ T cells specific for epitopes being present in both structural (core and envelope) and nonstructural (polymerase) proteins. Above this concentration, the detection of HBV-specific CD8+ cells in the circulation of chronic hepatitis B patients was more difficult; CD8+ T cells displayed phenotypic alterations (i.e., tetramer negativity) (34), and the frequency of direct detection was very low, with CD8+ T cells occasionally being detectable only after a round of in vitro expansion. More importantly, differences were detectable among CD8+ T cells specific for different epitopes. Core 18–27-specific CD8 cells, which are often dominant in patients with acute hepatitis B (Fig. 1) (21, 43), are most affected by increased levels of HBV replication. These cells cannot be detected in the circulation (either directly ex vivo or after in vitro expansion) when HBV DNA levels are $>10^7$ copies/ml, and we demonstrated that this lack of detec-

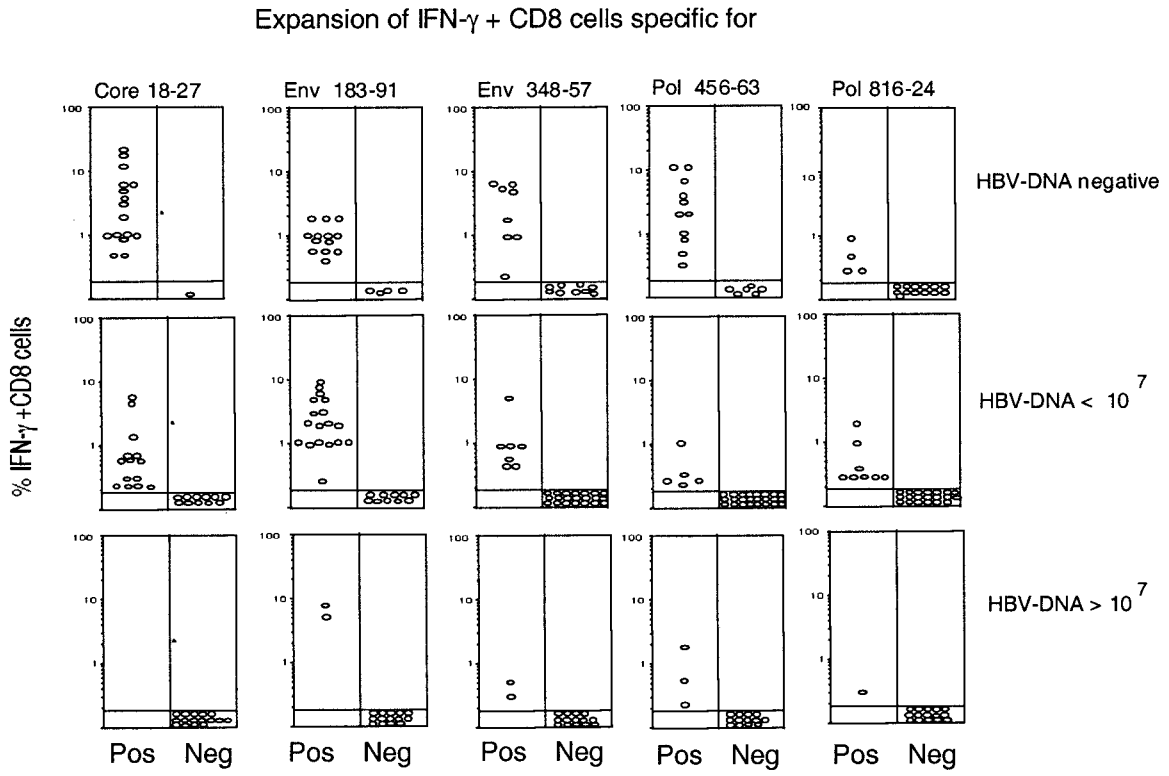

FIG. 5. Expansion of epitope-specific IFN-γ-producing $CD8^+$ T cells in relation to HBV DNA levels. The percentages of IFN-γ-producing $CD8^+$ T cells specific for the indicated HBV epitopes present in the T-cell lines generated after in vitro peptide stimulation of PBMC were grouped in relation to the HBV DNA levels present at the time of the assays. Data include "positive" (Pos) experiments (presence of peptide-specific IFN-γ-producing $CD8^+$ T cells) and "negative" (Neg) experiments (absence of peptide-specific IFN-γ-producing $CD8^+$ T cells) performed for patients studied longitudinally. HBV DNA levels are given in copies per milliliter.

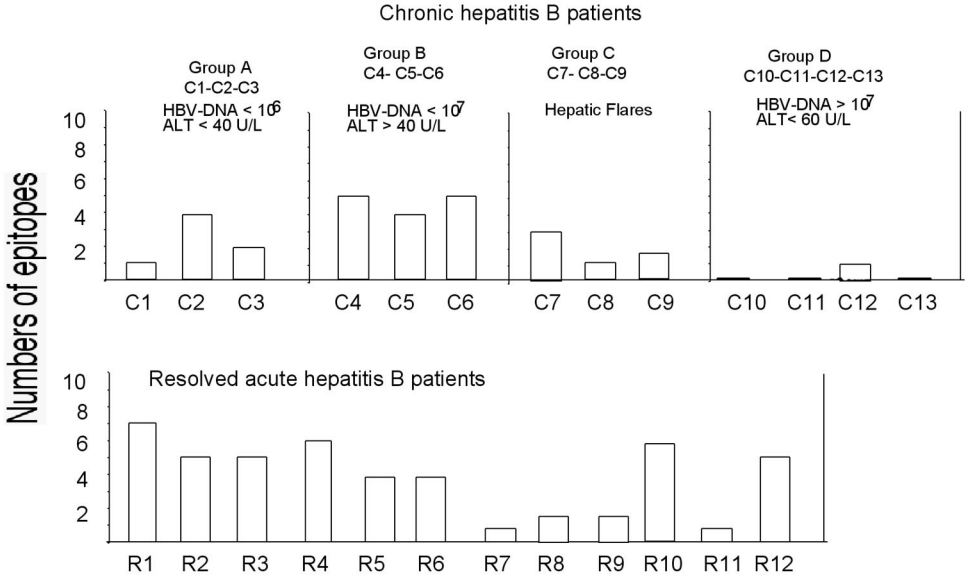

FIG. 6. Numbers of HBV epitopes recognized by patients with chronic or resolved HBV infections. Bars indicate the numbers of peptides recognized by each patient for the 11 HBV epitopes tested. PBMC from the indicated patients with chronic or resolved HBV infections were stimulated with 11 HBV peptides representing known HLA-A2-restricted epitopes (Table 2). After 8 to 10 days of in vitro expansion, the short-term cell lines were tested for the presence of peptide-specific IFN-γ-producing $CD8^+$ T cells. HBV DNA levels are given in copies per milliliter; ALT levels are given in units per liter (U/L).

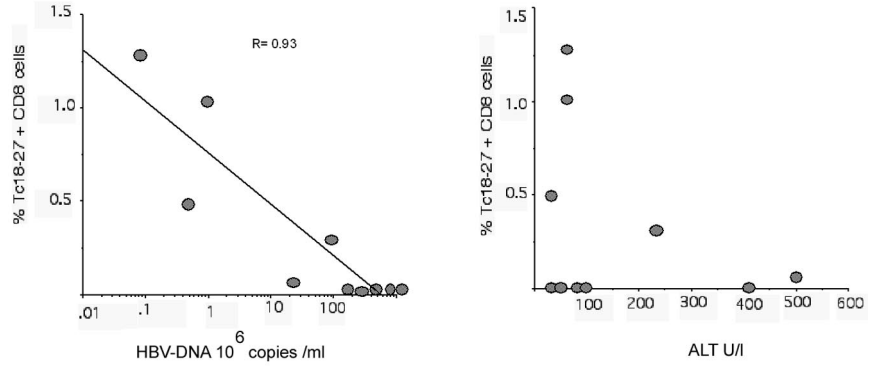

FIG. 7. Frequencies of intrahepatic core 18–27-specific CD8+ T cells in relation to HBV DNA and ALT levels. Percentages of tetramer core 18–27-specific (Tc18-27) CD8+ T cells present in lymphocytes purified from liver biopsy specimens were plotted in relation to the HBV DNA and ALT levels (units per liter [U/l]) present at the time of liver biopsy. Results obtained for 10 different patients are shown. There was a strong negative correlation between HBV DNA and core 18–27-specific CD8+ T cells (r value determined by the Spearman correlation test, -0.93; $P = 0.0001$) but no correlation between ALT and core 18–27-specific CD8+ T cells ($r = 0.23$; $P = 0.53$).

tion within the circulatory compartment is not caused by preferential intrahepatic localization of core 18–27-specific CD8+ T cells. The frequency of core 18–27-specific CD8+ T cells within the liver is also inversely proportional to the level of HBV replication. Except for a single case (patient C10), HBV infecting patients at concentrations of $>10^7$ copies/ml did not show within the core 18–27 sequence mutations which might abolish core 18–27-specific CD8+-T-cell expansion. Furthermore, we did not find any evidence for a progressive selection of low-avidity core 18–27-specific CD8+ T cells in chronically infected patients with HBV DNA levels of $<10^7$ copies/ml. Thus, the absence of circulating core 18–27-specific CD8+ T cells associated with HBV DNA levels of $>10^7$ copies/ml might be due to deletion caused by a high level of antigen (14, 35) or by an inherent inability to mount a CD8+-T-cell response against this epitope.

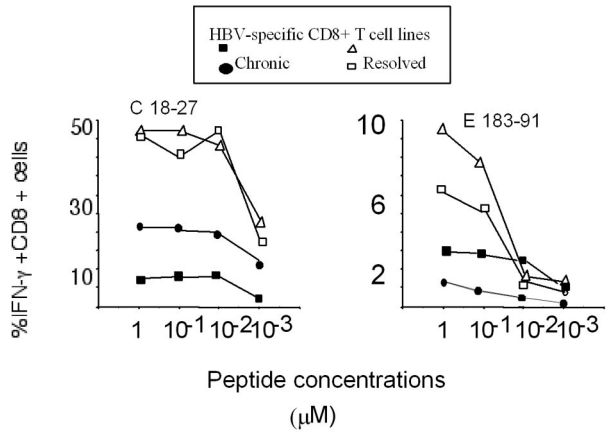

FIG. 8. Functional T-cell avidity of HBV epitope-specific CD8+ T cells. Various T-cell lines specific for the indicated HBV epitopes and produced in patients with resolved infections or chronic infections were stimulated with various concentrations of peptides. Core 18–27- and env 183–191-specific CD8+ T cells were derived from patients R1 and R2 (with resolved infections). Core 18–27-specific CD8+ T cells were derived from chronically infected patients C4 and C5; env 183–191-specific CD8+ T cells were derived from patients C5 and C7. Frequencies of IFN-γ-producing CD8+ T cells activated by the indicated peptide concentrations are shown.

Envelope- and polymerase-specific CD8+ T cells are instead the only cells which can be demonstrated in patients with chronic hepatitis B and concentrations of HBV DNA of $>10^7$ copies/ml. Their ability to persist in the presence of a high level of HBV replication is associated with an apparent inability to exert antiviral function. We did not find viral mutations in the relevant envelope and polymerase epitopes in patients who had envelope- or polymerase-specific CD8+ T cells. More importantly, envelope-specific CD8+ cells are characterized by an altered phenotype (tetramer negativity) (34), and their indifference to the dynamic fluctuations of HBV DNA levels (Fig. 1a, patient C7) is suggestive of a tolerant state. The persistence of polymerase-specific CD8+ T cells could be the result of the low quantity of polymerase epitopes expressed in vivo by infected hepatocytes, as suggested by recent results obtained with the transgenic mouse model of HBV infection (14). Interestingly, polymerase-specific CD8+ T cells do not recognize target cells transfected with the HBV genome and producing whole HBV virions (2.2.15 cells) (N. Naoumov et al., unpublished data), while core- and envelope-specific CD8+ T cells are efficiently activated by them.

We cannot rule out the possibility that the different behaviors of the distinct CD8 T-cell epitopes are due not to their HBV protein derivation but to the intrinsic features of the epitopes (such as affinity of HLA-A2 binding, efficiency of presentation, availability of a T-cell repertoire, and cross-reactivity) (7, 8, 17). Nevertheless, we think that a number of pieces of evidence support the former possibility. First, the different behaviors of HBV-specific CD8+ T cells, according to their antigenic derivation, were shown recently for transgenic mice (14), and our results are in line with these findings. Second, the persistence of envelope- and polymerase-specific CD8+ T cells in patients with HBV DNA levels of $>10^7$ copies/ml is not restricted to a single epitope but encompasses different epitopes present in the same HBV proteins (34). Third, CD8+-T-cell responses specific for core epitopes and restricted to non-HLA-A2 molecules display behaviors identical to those of HLA-A2-restricted core 18–27-specific CD8+ T

cells. They are usually dominant in non-HLA-A2-positive patients with self-limited infection (4) and are only present in chronically infected patients with low HBV DNA levels (40).

We think that this extensive analysis of HBV-specific $CD8^+$ T cells in patients with chronic hepatitis B might be relevant to the tailoring of new immunotherapeutic approaches for hepatitis B treatment. It remains difficult to select the best HBV proteins for inclusion in a therapeutic vaccine. The fact that core-specific $CD8^+$ cells are associated, as shown both here and in other work (10, 15), with the control of HBV replication strongly suggests that core antigen should be included in a vaccine formulation. Nevertheless, association is not proof of a causative effect, and we should not forget that this association might be the consequence and not the cause of low HBV DNA levels. However, demonstration that the extent of the peripheral repertoire of HBV-specific $CD8^+$ cells is inversely proportional to the level of HBV replication reinforces the therapeutic approach of inhibiting HBV by using antiviral drugs before using vaccines to boost HBV-specific T-cell immunity (3). Our data suggest that HBV replication should be reduced to a level lower than 10^7 copies/ml in order to maximize the chances of vaccines to expand a broad repertoire of HBV-specific $CD8^+$ cells.

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