Hepatitis B Virus (HBV) Sequence Variation in Cytotoxic T Lymphocyte Epitopes is not Common in Patients with Chronic HBV Infection

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Abstract

It has been suggested that immune selection pressure exerted by the cytotoxic T lymphocyte (CTL) response could be responsible for viral persistence during chronic hepatitis B virus infection. To address this question, in the current study we compared the DNA and amino acid sequences of, and the CTL responses to, multiple HLA-A2-restricted CTL epitopes in the hepatitis B virus in several HLA-A2-positive patients with acute and chronic hepatitis. Our results indicate that the CTL response to these epitopes is barely detectable in the majority of patients with chronic hepatitis. Further, we show that the weak CTL response is not secondary to infection by mutant viruses lacking these epitopes, and we show that the CTL response did not select for escape mutants in any of these patients. We conclude that an ineffective hepatitis B virus specific CTL response is the primary determinant of viral persistence in chronic hepatitis and that immune selection of viral variants is not a common event in the majority of patients. (*J. Clin. Invest.* 1995. 96:1527-1534.) Key words: hepatitis B virus • immune response • mutation • cytotoxic T cell • viral escape

Introduction

The hepatitis B virus (HBV)[1] is a noncytopathic DNA virus with a small, circular DNA genome that causes acute and chronic liver disease and hepatocellular carcinoma. Patients with acute, self-limited HBV infection usually mount a vigorous and polyclonal cytotoxic T cell response, which is also multispecific, i.e., targeted against multiple epitopes in the viral nucleocapsid (1-4), envelope (5) and polymerase proteins (6). In contrast, a vigorous, polyclonal cytotoxic T lymphocyte (CTL) response is usually not readily detectable in the peripheral blood of patients with chronic HBV infection although HBV-specific T cells are present in the chronically inflamed liver and are thought to contribute to the pathogenesis of chronic hepatitis (7, 8). For these reasons it is likely that a strong, polyclonal CTL response to HBV plays an essential role in viral clearance and clinical recovery during acute hepatitis, and that viral persistence reflects a diminished CTL response that is not strong enough to clear the virus but is sufficient to maintain the spectrum of low grade inflammatory liver diseases designated as chronic hepatitis.

The host-virus interactions that facilitate viral persistence and cause chronic hepatitis, however, are not well defined. In infected newborns the diminished antiviral immune response is probably due to neonatal tolerance mechanisms, while immunogenetic, environmental, or virological factors could be responsible for the diminished immune response in chronically infected adults. Irrespective of the cause, however, a diminished CTL response that is permissive of viral persistence could, theoretically, select for the emergence of viral escape mutants that are even less visible to the already ineffective immune system and, thereby, contribute further to persistent infection. The likelihood of this process would be significantly enhanced if the CTL response was narrowly focussed and relatively strong (i.e., functionally monospecific) as has been reported to occur during lymphocytic choriomeningitis virus infection in transgenic mice that express a single T cell receptor specific for a single lymphocytic choriomeningitis virus (LCMV) epitope (9). Importantly, this scenario was recently reported in two patients with chronic HBV infection who produced a relatively strong, but narrowly focussed CTL response to a single epitope in the hepatitis B virus (10, 11).

Several other groups (12-14) have recently described HBV sequence heterogeneity in chronically infected patients, and they have suggested that the sequence heterogeneity could reflect immune selection. However, a systematic comparative analysis of the T cell response to epitopes potentially encoded by these sequences was not performed in these patients, so the significance of those findings is uncertain.

The current study was performed to compare these virological and immunological events simultaneously in a group of patients with chronic HBV infection to clarify the importance of CTL selection pressure and viral escape mutations in this disease.

Methods

Patient population. 12 HLA-A2-positive patients with chronic hepatitis B and 6 HLA-A2-positive patients with acute hepatitis B were studied. The diagnosis of acute hepatitis B was based on clinical and biochemical evidence of acute liver injury according to standard diagnostic criteria, i.e., jaundice and elevated values of alanine aminotransferase activity at least 20-fold greater than the upper limit of normal, together with serological evidence of acute HBV infection, i.e., hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg) and IgM anti-HBc antibody (IgM HBc-Ab), and the absence of serologic evidence of hepatitis delta or hepatitis C virus infection (Abbott Laboratories, North Chicago, IL). All patients recovered completely with normalization of serum transaminases and clearance of HBsAg and HBeAg within 4 mo of initial diagnosis. Blood samples for serum and PBMC analysis were taken at the time of clinical presentation, when diagnosis of acute HBV

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1. *Abbreviations used in this paper:* CTL, cytotoxic T lymphocyte; HBV, hepatitis B virus; LCMV, lymphocytic choriomeningitis virus.

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Table I. Clinical, Serological, and Virological Features of the Patients Studied

Patient*	Age	Sex‡	HLA	HBsAg	HBeAg	antiHBc	antiHBs	antiHBe	ALT§
	yr								*U/l*
C-1	35	M	A2A24 B44B67	pos.	pos.	pos.	neg.	neg.	316
C-2	38	M	A2A30 B44B13 Cw6	pos.	neg.	pos.	neg.	neg.	38
C-3	36	M	A2A69 B41B52	pos.	pos.	pos.	neg.	neg.	139
C-4	36	M	A2 B13B7801 Cw7	pos.	pos.	pos.	neg.	neg.	115
C-5	49	M	A2 A23 B44	pos.	pos.	pos.	neg.	neg.	49
C-6	66	M	A2A1 B8B44	pos.	pos.	pos.	neg.	neg.	123
C-7	21	F	A2A26 B46B72 Cw3Cw1	pos.	neg.	pos.	pos.	neg.	105
C-8	57	M	A2A68 B59B44 Cw5Cw7	pos.	pos.	pos.	neg.	neg.	78
C-9	n.k.‖	M	A2 B62 Cw8	pos.	pos.	pos.	pos.	neg.	79
C-10	53	M	A2A34 B8B27 Cw7	pos.	pos.	pos.	neg.	neg.	120
C-11	50	M	A2 B27B46 Cw1	pos.	pos.	pos.	neg.	neg.	54
C-12	n.k.	F	A2 B7B39	pos.	neg.	pos.	neg.	neg.	24
A-1	26	M	A2A1 B8Bw58 Cw7	pos.	pos.	pos.	n.d.¶	n.d.	2082
A-2	22	M	A2Aw69 Bw53 Cw4	pos.	pos.	pos.	n.d.	n.d.	3918
A-3	38	M	A2A24 B51B53 Cw1	pos.	pos.	pos.	neg.	neg.	844
A-4	19	n.k.	A2A24 B7B27 Cw2Cw7	pos.	n.d.	pos.	n.d.	n.d.	2044
A-5	34	M	A2A68 B44B54 Cw5Cw7	pos.	neg.	pos.	neg.	neg.	3822
A-6	22	F	A2A74B62B57Cw3Cw6	neg.	neg.	pos.	neg.	pos.	371

* Patients with chronic hepatitis B are identified by a C preceding the patient number, patients with acute hepatitis B with an A; ‡ M, male; F, female; § ALT, alanine aminotransferase, normal range 0–45 U/liter; ‖ n.k., not known; ¶ n.d., not done; pos., positive; neg., negative.

infection was first established. All patients with chronic hepatitis B were repeatedly serologically positive for HBsAg for at least 6 mo and in selected cases (C-1, C-3, C-6) for more than 3 yr. Eight of them displayed elevated transaminases of twice the upper limit of the normal range or higher, while four patients (C-2, C-5, C-11, C-12) had mildly elevated or normal transaminases when blood samples for sequencing and CTL analysis were taken. The clinical, virological and serological characteristics of these patients are summarized in Table I.

All subjects studied were repeatedly negative for antibodies to HCV and HIV. HLA typing of PBMC was performed by complement microcytotoxicity using HLA typing trays purchased from One Lambda (Canoga Park, CA) (Table I). The study protocol was approved by the Human Subjects Committee at the Scripps Clinic and Research Foundation.

CTL analysis. PBMC were separated on Ficoll-Histopaque density gradients (Sigma Chemical Co., St. Louis, MO), washed three times in HBSS (GIBCO Laboratories, Grand Island, NY), resuspended in RPMI 1640 (GIBCO Laboratories) supplemented with L-glutamine (2 mM), gentamycin (10 mM), and 10% heat-inactivated human AB serum and plated in a 24-well plate at 4×10^6 cells/well. rHBcAg (Biogen, Cambridge, MA) was added to the cell cultures at 1 mM and the synthetic peptides at 10 μM. The peptides used have been described as CTL-epitopes in previous studies (3, 5, 6, Chisari, F. V., unpublished observations). On days 3 and 10, 1 ml of RPMI 1640 with 10% human AB serum and rIL-2 (Hoffmann-La Roche, Inc., Nutley, NJ) at 10 U/ml final concentration was added to each well. On day 7, the cultures were restimulated with peptide, rIL-2, and irradiated (3,000 rad) autologous feeder cells. In a subset of chronically infected patients the CTL response to an additional panel of 32 HBV-derived peptides with good HLA-A2–binding affinity was tested using a modification of the foregoing stimulation strategy as previously described (10). In all cases, cultures were tested for cytotoxic activity on day 14 against JY-EBV incubated overnight with synthetic peptides at 10 μM, labeled with 100 μCi of ^{51}Cr (Amersham Corp., Arlington Heights, IL) for 1 h and washed four times with HBSS. Cytolytic activity was determined in a standard 4-h ^{51}Cr release assay using U-bottomed 96-well plates containing 5,000 targets/well. All assays were performed in duplicate. Percent cytotoxicity was determined from the formula: $100 \times$ [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Maximum release was determined by lysis of targets by detergent (1% Triton X-100; Sigma Chemical Co.). Spontaneous release was < 20% of maximal release in all assays. The assay was considered positive if the specific ^{51}Cr release from target cells containing antigen was ≥ 15% higher than the nonspecific ^{51}Cr release from antigen negative target cells, and the nonspecific lysis was < 15% of maximum.

DNA-analysis. HBV-DNA was extracted from the serum as previously described, dissolved in TE buffer (15) and amplified in a programmable thermal cycler (Twin Block System; ERICOMP, Inc., San Diego, CA) by nested PCR. To amplify the region containing epitope Env250-258, Env260-269, Env335-343, Env348-357, Env370-379, Pol455-463, and Pol551-559 primer combination 1 or 2 was used. To amplify the region containing epitope Core18-27 primer combination 3 or 4 was used (Table II). For each PCR the following program was used: 94°C for 4 min followed by 40 cycles of denaturation at 93°C for 5 min, annealing at 56°C for 2 min, and elongation at 72°C for 3 min. The last cycle was followed by a 4-min extension step at 72°C. The PCR products were analyzed by ethidium bromide–stained agarose gel electrophoresis. To eliminate sources of DNA contamination, DNA extraction and first PCR were performed in a separate room from the one used for handling of the amplified PCR products. Pipetting devices were kept separately and plugged pipette tips were used. All sera were divided into small aliquots under sterile conditions in a laminar flow hood and a separate aliquot was used for each analysis. All extractions and amplifications were performed in parallel with relevant negative and positive controls which consisted of DNA derived from the serum of healthy, uninfected individuals and HBV-positive patients. Contamination controls consisted of water added to the PCR mixture instead of DNA.

Direct sequencing of the PCR-products was performed with the PCR-product sequencing kit (Sequenase™; Amersham/USB, Arlington Heights, IL). Primer HBV-370S was used to sequence epitope Env250-258, Env260-269, Pol455-463 in sense direction, primer HBV-536S to sequence epitope Env335-343, Env348-357, Env370-378, and Pol551-559 in sense direction. Primer HBV-660AS was used to sequence epitope Env250-258, Env260-269, Pol455-463 in antisense direction and primer HBVSIAN to sequence epitope Env335-343, Env348-357, Env370-378, Pol551-559 in antisense direction. Epitope Core18-27 was

Table II. Oligonucleotide Sequence of Primers

	Sequence	Position
Primer combination 1:		
External primers		
HBV-246S	5′-GAGTCTAGACTCGTGGTGGA	246–265
HBV-1086AS	5′-GCGAGAAAGTGAAAGCCTG	1086–1105
Internal primers		
HBV-370S	5′-TATCGCTGGATGTGTCTGC	370–388
HBV-HBVSIAN	5′-TTTAGAGAGTAACCCCATCT	851–870
Primer combination 2:		
External primers		
HBV2830S	5′-GAACAAGATCTACAGCATGG	2830–2849
HBV-1086AS	5′-GCGAGAAAGTGAAAGCCTG	1086–1105
Internal primers		
HBV-150S	5′-AACATGGAGAACATCACATC	150–169
HBV-HBVSIAN	5′-TTTAGAGAGTAACCCCATCT	851–870
Sequencing primers:		
Sense:		
HBV-150S	5′-AACATGGAGAACATCACATC	150–169
HBV-370S	5′-TATCGCTGGATGTGTCTGC	370–388
HBV-536S	5′-TCAAGGAACCTCTATGTATC	536–555
Antisense:		
HBV-353AS	5′-AGCGATAACCAGGACAAGTT	353–372
HBV-660AS	5′-CTGAGCCAGGAGAAACGG	660–678
HBV-HBVSIAN	5′-TTTAGAGAGTAACCCCATCT	851–870
Primer combination 3:		
External primers		
HBV-XISE	5′-CAAGGTCTTACATAAGAGGA	1641–1660
HBV-2860AS	5′-CAGAGGATTGCTGGTGGA	2861–2878
Internal primers		
HBV-X2SE	5′-CTCTTGGACTCTCAGCAATG	1661–1680
HBV-2627AS	5′-CCTGGCAGGCATAATCAATT	2627–2646
Primer combination 4:		
External primers		
HBV-X2SE	5′-CTCTTGGACTCTCAGCAATG	1661–1680
HBV-CBHINT	5′-TTGCCTGAGTGCAGTATGGTGAGG	2046–2069
Internal primers		
HBV-PCO	5′-GGGAGGAGATTAGGTT	1742–1757
HBV-COR	5′-AACATTGAGATTCCCGA	2432–2448
Sequencing primers:		
Antisense:		
HBV-CBHINT	5′-TTGCCTGAGTGCAGTATGGTGAGG	2046–2069
HBV-NCO	5′-CGGAAGTGTTGATA	2317–2330
HBV-COR	5′-AACATTGAGATTCCCGA	2432–2448

sequenced with primer HBV-CBHINT and the rest of the Core region was sequenced with primers HBV-COR and HBV-NCO in antisense direction. The predominant sequence was defined by the major band in the sequencing lanes when PCR-products were sequenced or by the sequence of > 50% of 5 to 12 independent clones. As shown by others (16) a viral subpopulation as low as 10% of the total population can be detected by direct sequencing of PCR-products. In selected cases, the PCR product was subcloned into the pCR™-vector with the TA Cloning Kit (Invitrogen, San Diego, CA). Cultures from individual colonies were grown overnight, plasmid was isolated with the QIAGEN Miniprep Kit (QIAGEN, Chatsworth CA) and 6–12 clones derived from at least two independent PCR reactions were sequenced.

Each patient's sequence was compared to the HBV sequences HPBAYWCI, HPBHBVAA, HPBVAYWE, HPBV, HPBVAYWC, HPBAYW, HPBADWZCG, HPBADW, HPBADW1, HPBHEPB, HPBADW2, HPBADWZ, HPBADW3, HPBADRCG, HPBADRC, HPBADRA, HPBCGADR, HPBVADRM, HPBADR, and HPBAYR published in GenBank and to their consensus sequence. If patients were studied sequentially the HBV sequence was compared to the sequence derived from earlier serum samples from the same patient.

Results

Analysis of the CTL response to a panel of T cell epitopes in acute and chronic HBV infection. In this study we analyzed the CTL response to and the deduced amino acid sequence of 8 independent CTL epitopes in 6 patients with acute hepatitis and 12 patients with chronic hepatitis. The epitopes were chosen because they are located in three different HBV proteins,

Table III. The Epitopes

Epitope	Peptide sequence*	Conservation in GenBank‡	Conservation in Acutes§	Conservation in Chronics§	Alternative peptide sequences in GenBank databases‖	Alternative peptide sequences as found in patients¶	Patients**
Core18–27	FLPSDFFPSV	45%	100%	92%	FLPSDFFPSI YLPSDFFLYV	FLPSDFFPSI	C-9
Env250–258	LLLCLIFLL	100%	100%	100%	none	none	
Env260–269	LLDYQGMLPV	90%	83%	100%	LLDYQGMLHV	none	
Env335–343	WLSLLVPFV	100%	100%	100%	none	none	
Env348–357	GLSPTVWLSV	75%	33%	42%	GLSPTVWLSA	GLSPTVWLSA	A-2,A-4,A-5,C-3,C-5,C-6,C-7,C-8,C-11
					GLSPTVWSSV	GLSPTVWPSA	C-4
Env370–379	SIVSPFIPLL	10%	50%	33%	SILSPFLPLL	SILSPFLPLL	C-2
					SIVSSFIPLL	NILSPFLPLL	C-6
					TILSPFLPLL	NILSPFIPLL	C-4,C-5,C-8,C-9,C-10
					NILSPFMPLL	NIVSPFIPLL	A-2
						SIVSPFLPLL	A-1,A-4,C-7
Pol455–463	GLSRYVARL	55%	100%	92%	GLPRYVARL	GLPRYVARL	C-3
Pol551–559	YMDDVVLGA	90%	100%	100%	YMDDMVLGA	none	

* Amino Acid Sequence of epitope peptides used to stimulate CTL responses in PBMC. ‡ Frequency (%) at which the epitope peptide sequence is present in the HBV isolated published in GenBank. § Frequency (%) at which the epitope peptide sequence is present in the patients studied. ‖ Alternative peptide sequences at the corresponding locations in the GenBank database. ¶ Actual viral peptide sequences detected in patients included in this study. ** Patients in whom the variant sequences were identified.

namely core, envelope, and polymerase, and because they have been shown to expand in vivo–primed polyclonal CTL from patients with acute hepatitis B (1, 5, 6). As we have previously reported, this CTL response is multispecific, i.e., targeted against multiple epitopes in all viral proteins, and it can persist for 6–18 mo after recovery from acute hepatitis B infection, and cytotoxic T lymphocytes against all of these epitopes have been shown to be CD8 positive and HLA-A2 restricted (1, 5, 6, Chisari, F. V., unpublished observations). The degree of sequence conservation within these epitopes varies from 10–100% of the sequences present in the GenBank Data Base (Table III).

As shown in Fig. 1, all of the 6 acutely infected patients responded to two or more of the epitopes tested. A total of 23 CTL responses were detected out of a possible 43 (53%) in this group of patients. CTL specific for these epitopes have previously been shown to recognize target cells pulsed with very low concentrations (0.001 μM) of the inducing peptide, but they do not recognize irrelevant peptides even at high concentrations (10 μM) (1, 5, 6). Their relevance during an acute infection is supported by the fact, that such CTL have also been shown to lyse target cells presenting endogenously processed antigen (1, 5, 6).

In contrast, 8 of the 12 chronically infected patients failed to respond to any of the epitopes, and the remaining 4 patients produced only weak responses to a single epitope, such that only 5 out of 96 (5%) possible CTL responses were observed in these chronically infected patients. To examine the possibility that chronically infected patients responded to a different set of peptides from the acutely infected patients, we stimulated PBMC from a subset of these patients with an extended panel of HBV-derived peptides with high to intermediate HLA-A2 binding affinity in the range which we have previously shown to be required for immunogenicity (17). We have previously shown this panel of peptides to be nonstimulatory to acutely infected patients (17). As shown in Table IV, no CTL responses were generated to any of these peptides by these patients.

Analysis of the T cell epitope sequence of the infecting virus in acute and chronic HBV infection. As stated above, a narrowly focused CTL response, that might exert selective pressure on the virus, was observed in 5 out of 96 CTL assays in chronically infected patients (Fig. 1). In each instance, however, only prototype epitope sequences matching the ones in the peptides were observed (Table V). Similarly, prototype epitope sequences were present in 23 out of 23 instances (100%) where a strong CTL response was observed in the acute HBV patients. This suggests that CTL pressure was not sufficient to select for escape mutants at these loci in these patients. Additionally, since an equal fraction of the examined epitopes ($\sim$ 20%) were different from the stimulating peptide in both groups of patients, and since this was always associated with nonresponsiveness at these loci, it is possible that the patients had not been primed in vivo to these epitopes. This should not be a cause of persistent infection, however, since it was seen in only a few isolated and randomly distributed epitopes, and it was as common in patients who cleared the virus as it was in those who did not (Table III).

CTL response to alternate viral epitope sequences. To understand whether the patients infected by viral variants who did not respond to the prototype peptide could respond to the variant peptides, we stimulated the PBMC from patient C-2 and C-6 who failed to respond to Env 370-378 with the peptides corresponding to the viral sequence with which they were infected (Table III). In both peptides the HLA-A2–binding motif was still present. No specific cytotoxicity was observed against the prototype or the variant peptides in either patient (not shown) suggesting that the variant sequences were not selected by immune pressure and that they were either present in the virus at the time of infection or they were selected for other reasons. Alternatively, the CTL response against the prototype sequence could have vanished and the new variant epitope could be unable to stimulate a new CTL response.

Sequential analysis of epitope sequence and CTL response during chronic HBV infection. Patients C-4, C-6, and C-3 with

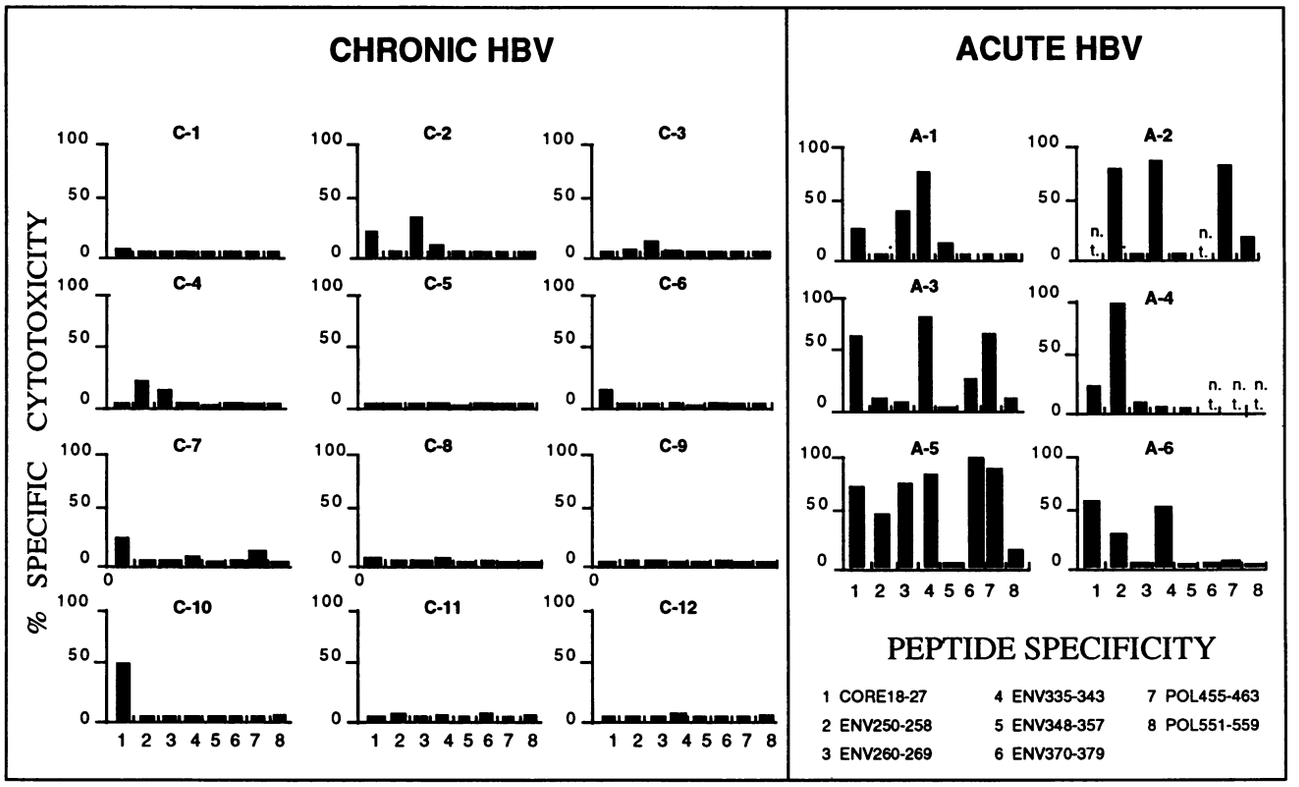

Figure 1. The CTL response in acute and chronic HBV infection: HBV-specific CTL response in patients with chronic hepatitis (*C-1–C-9*) and acute hepatitis (*A-1–A-9*). PBMC were stimulated with 10 μg of the peptides indicated for 2 wk as described in Methods, and tested in a 4-h ^{51}Cr release assay against JY target cells prepulsed overnight with the same peptide. Peptide-specific cytotoxicity was measured by subtracting the ^{51}Cr release by JY target cells not prepulsed with the peptide from the ^{51}Cr release by JY target cells prepulsed with 10 μM of the peptide. Results shown represent percent specific lysis in a 4-h ^{51}Cr release by JY target cells prepulsed with 10 μM of the peptide at an Effector: Target Ratio of 50:1 (n.t., not tested).

chronic HBV infection were followed over 10, 29, and 55 mo, respectively. In patient C-3 a sequence change was found in the HLA-A2.1–binding residue of the core18-27 epitope of serum-derived HBV-DNA, so this patient was analyzed sequentially (Table VI). Synthetic peptides corresponding to the original and the alternate sequence were used to stimulate peripheral blood lymphocytes and to test for specific cytotoxicity against JY-EBV cells pulsed with the corresponding peptide. No immune response to either the original or alternate sequence was observed.

Analysis of sequence variability in the core mutation "cluster" region in chronically infected patients. To compare the degree of sequence heterogeneity in our patients with that reported previously in patients with active liver disease from Japan (13) and China (14), we sequenced the first 139 codons of the core region which contain two of the three mutation "cluster" regions defined by those investigators (i.e., codons 48–60 and 84–101). We compared the HBV nucleotide sequence derived from each patient with the HBV sequences HPBAYWCI, HPBHBVAA, HPBVAYWE, HPBV, HPBVAYWC, HPBAYW, HPBADWZCG, HPBADW, HPBADW1, HPBHEPB, HPBADW2, HPBADWZ, HPBADW3, HPBADRCG, HPBADRC, HPBADRA, HPBCGADR, HPBVADRM, HPBADR, and HPBAYR published in GenBank. The comparison with isolate HPBADR and the previously published data is shown in Fig. 2. In contrast to the previous reports (13, 14) in Eastern patients we do not find clustered mutations in codon 48–60 and 84–101. The number of divergent amino acids is even less in our patient group if subtype HPBADW, which is more frequent in Western patients, is chosen for amino acid comparison. In this case there will be no amino acid differences at position 75, 77, 87, and 91 of the core protein (marked by an asterisk in Fig. 2).

Comparison of sequence variability in intraepitope and interepitope regions in acute and chronic HBV infection. When each patient's HBV sequence was compared to the HBV sequences published in GenBank no complete match was found. Each patient-derived HBV sequence shared between 89 and 100% of the nucleotides with each of the GenBank HBV sequences. To standardize the analysis of intra- and interepitope variability, the patient-derived HBV sequences were compared to the consensus sequence of the GenBank HBV isolates. As shown in Table VII, the sequence variability in both intra- and interepitope regions in the chronically infected patients was the same as in the acutely infected patients. Importantly, at these epitope sites in both patient populations the intraepitope regions display no greater sequence variability than the interepitope region of the virus suggesting that CTL selection pressure at these epitope sites is absent in these patients.

Discussion

In this study, we demonstrated that CTL escape mutations are not commonly observed in viral sequences in patients with

Table IV. Analysis of an Extended Panel of Peptides in Patients with Chronic HBV

Protein	Amino acid location	Epitope sequence	HLA-A2.1-binding affinity	Percent conserved*	No. Positive/No. Tested CTL-assays‡	Patients§
Env	371–379	ILSPFLPLL	2.9000	65	0/24	0/3
Core	118–126	LVSFGVWI	2.7000	90	0/24	0/3
Env	338–347	LLVPFVQWFV	1.6000	95	0/24	0/3
Pol	527–535	LLAQFTSAI	0.7100	100	0/48	0/3
Env	259–268	VLLDYQGMLPSV	0.7000	90	0/24	0/3
Env	360–368	MMWYWGPSL	0.4100	85	1/24	1/3
Env	249–258	ILLLCLIFLL	0.3000	100	0/24	0/3
Env	348–357	GLSPTVWLSV	0.2800	75	0/52	0/4
Core	18–27	FLPSDFFPSI	0.2100	50	0/24	0/3
Pol	655–663	ALMPLYACI	0.2000	100	0/24	0/3
Core	59–71	ILCWGELMTL	0.1900	45	0/24	0/3
Env	188–195	VLQAGFFLL	0.1500	95	0/24	0/2
Pol	63–71	GLYSSTVPV	0.1500	60	0/24	0/2
Pol	504–512	HLYSHPIIL	0.1300	84	0/40	0/4
Env	259–267	VLLDYQGML	0.1100	95	0/24	0/2
Env	378–388	LLPIFFCLWV	0.1000	100	0/24	0/2
Env	260–270	LLDYQGMLPV	0.0850	90	0/24	0/2
Env	359–268	WMMWYWGPSL	0.0840	85	0/24	0/2
Pol	816–824	SLYAVSPSV	0.0780	56	0/40	0/4
Env	251–259	LLCLIFLLV	0.0490	100	0/40	0/4
PreCore	2–10	QLFHLCLII	0.0390	88	0/24	0/2
Env	249–257	ILLLCLIFL	0.0350	100	0/24	0/2
Env	175–184	LLVLQAGFFL	0.0310	90	0/24	0/2
Env	377–385	PLLPIFFCL	0.0310	100	0/24	0/2
Env	248–257	FILLLCLIFL	0.0280	80	0/40	0/4
Core	100–108	LLWFHISCL	0.0240	90	0/40	0/4
Core	139–148	ILSTLPETTV	0.0220	100	0/40	0/4
Pol	773–782	ILRGTSFVYV	0.0160	89	0/40	0/4
Env	176–184	LVLQAGFFL	0.0150	90	0/40	0/4
Pol	765–773	LLGCAANWI	0.0140	89	0/40	0/4
Core	99–108	QLLWFHISCL	0.0130	90	0/40	0/4
Pol	424–432	NLSWLSLDV	0.0130	90	0/40	0/4

* Percentage of HBV sequences containing the precise epitope in GenBank database; ‡ CTL assay rated positive, if specific cytotoxicity > 15% lysis; § patient rated positive if one CTL assay was rated positive.

chronic HBV infection. To examine this question, we compared the DNA sequence of, and the CTL response to, eight HLA-A2–restricted CTL epitopes in the HBV nucleocapsid, envelope, and polymerase proteins in 18 HLA-A2–positive patients with acute ($n = 6$) and chronic hepatitis B ($n = 12$). We report that the absence of a detectable CTL response in the majority of patients with chronic hepatitis B virus infection in the current study is not due to infection by viruses that contain variant CTL epitopes. In the few instances where a narrowly focused CTL response was present in the chronically infected patients, it did not lead to the emergence of CTL escape mutants. Nucleotide and amino acid sequences were not more variable within the CTL epitopes than within viral regions external to the epitopes, and the entire HBV core protein displayed the same degree of amino acid heterogeneity as the rest of the sequenced virus in these patients. Furthermore, the amino acid sequence heterogeneity of the virus present in the persistently infected patients was no greater than that seen in acutely infected patients who successfully cleared the virus. Finally, in a chronically infected patient who developed an amino acid change in the HLA-A2–binding residue of one of these epitopes, no immune response was detectable to either the original sequence or to the emerged variant indicating that variation can occur within well-defined CTL epitopes in the absence of immune selection pressure.

We conclude that the weak HBV-specific CTL response we observed in these chronically infected patients is the primary determinant of viral persistence, and that in general the CTL response is too weak in most chronically infected patients to

Table V. Analysis of CTL Epitope Sequence and CTL Response

Category	CTL response	Peptide sequence: Indentical		Peptide sequence: Nonidentical	
		Responders	*Nonresponders*	*Responders*	*Nonresponders*
Acute	23/43	23/23	16/20	0/23	4/20
	(53%)	(100%)	(80%)	(0%)	(20%)
Chronic	5/96	5/5	74/91	0/5	17/91
	(5%)	(100%)	(81%)	(0%)	(19%)

Table VI. Epitope Sequence Change and Immune Response

	Sequencing analysis	Immunological analysis	
	HBV-epitope	Specific cytotoxicity	
Date	sequence in serum	FLPSDFFPSI*	FLPSDFFPSV*
11/06/87	FLPSDFFPSI	0	n.t.
02/05/88	I	0	n.t.
09/22/89	I	1	n.t.
12/15/89	I	2	n.t.
08/01/90	I	n.t.	0
08/30/91	V	0	0
04/17/92	V	0	2
06/08/92	V	3	3

* Peptide used to expand CTL and to pulse JY-EBV used as targets in the CTL assay. n.t., not tested.

Table VII. Sequence Variability in Different Regions of the Hepatitis B Virus

	Amino acids analyzed*	Percent homology with GenBank HBV consensus sequence
Chronic HBV patients		
Region with epitopes	912	97.8
Region without epitopes	4580	94.4
Acute HBV patients		
Region with epitopes	456	98.7
Region without epitopes	794	95.6

* The number of amino acids analyzed refers to all of the 12 patients with chronic hepatitis B and all of the 6 patients with acute hepatitis B, respectively. The number of amino acids in regions without epitopes also considers translations according to different open reading frames.

select for the emergence of escape mutants at the epitope sites studied. Since we limited our analysis to eight epitopes previously defined in patients with acute hepatitis we cannot completely exclude the possibility that CTL responses to different epitopes might occur in patients with chronic infection and that these responses might select CTL escape mutants. We believe, however, that CTL escape mutations are unlikely to be a frequent event for several reasons: first, all the epitopes we studied are commonly recognized by patients with acute hepatitis B who successfully clear the virus. Second, we failed to observe CTL responses to an extended panel of 32 additional HBV-derived peptides that bind well to HLA-A2. Moreover, the existence of a completely different set of subdominant epitopes in patients with chronic HBV infection has not been demonstrated at this point in time. Third, in the two previously published cases (10, 11) in which viral escape may have indeed occurred, the narrowly focussed immune response targeted against one of these epitopes was uncommonly strong for chronically infected patients in our experience. Fourth, the rarity of CTL escape at the epitope described in these patients is underscored by the absence of variation in that sequence in the 20 HBV isolates catalogued in the GenBank database. Nonetheless, until it becomes technically possible to study the CTL response to full length viral proteins in HBV-infected individuals, the possibility that CTL responses to currently unidentified epitopes can effectively select for escape variants remains possible. Based on the currently available data, however, we propose that selection of CTL escape mutants is probably an unusual event during chronic HBV infection and that it probably occurs only when the CTL response is stronger than usual but narrowly focussed (8, 9).

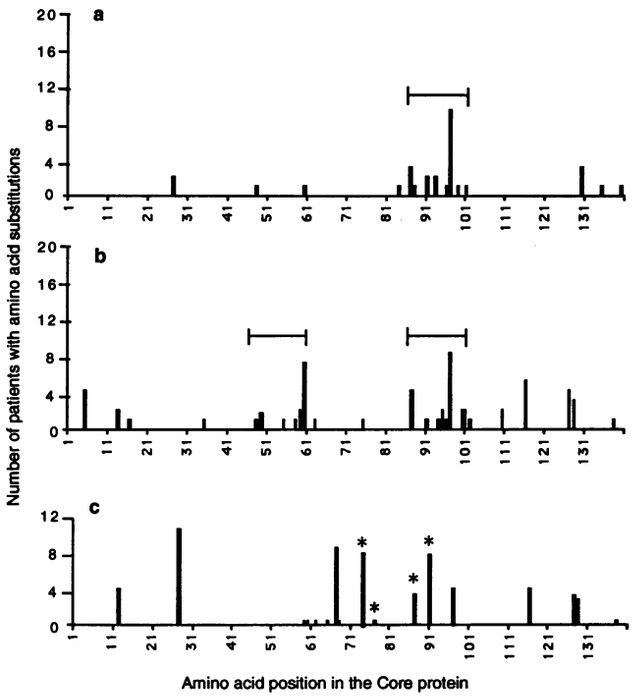

Figure 2. Amino acid variability of the Core protein. The number of patients with amino acid substitutions at each position of the Core protein is given for the study of Ehata et al. (*a*) (13), Chuang et al. (*b*) (14), and this study (*c*). The amino acid sequence is compared to the HPBADR subtype to allow comparison with the two previously published studies (13, 14). As the current study (*c*) analyzes a Western patient population, which is most likely infected by the HPBADW subtype, amino acids that do not match with HPBADR, but do match with HPBADW are marked with an asterisk. The mutation cluster regions (amino acid 48–60 and 84–101) are denoted by a horizontal bar above the corresponding positions.

The current data suggest that if CTL "escape" mutations occur in HBV, they could contribute to its persistence, but they probably do not represent its primary cause in the majority of patients where the precipitating factor would appear to be a weak immune response. Indeed, the absence of viral escape mutants in the current study is additional evidence that the CTL response in most chronically infected patients is too weak to select for variants.

Several other mechanisms might explain viral persistence in chronically infected patients. These include neonatal tolerance, immunological exhaustion by large viral loads, infection of immunologically privileged sites, modulation of recognition molecules on the surface of infected cells, etc. We favor the idea that the outcome may in fact be determined very soon after infection by the relative kinetics of viral spreading and induction of the immune response. According to this scenario a rapid and vigorous immune response would lead to viral clearance. In contrast, if the immune response is relatively slow to begin, the number of virus-infected hepatocytes may become so high that immune-mediated viral clearance could be impossible even if

the late-developing CTL response is vigorous. In this setting, viral mutations that abrogate or antagonize T cell recognition could be selected, but even under these circumstances the immune response to all of the remaining viral epitopes would have to be unable to clear the virus for selection to occur.

Finally, the current data demonstrate that comparisons between individual single viral isolates and consensus sequences are not likely to reflect mutations that have occurred in that patient unless they are located within otherwise conserved regions of the virus. Furthermore, these results illustrate that the only definitive evidence of viral escape mutation is demonstration of an amino acid sequence change in a specific epitope in two sequential samples from an individual patient combined with loss of recognition of that epitope by CTL derived from the same patient. Unless this kind of combined analysis of viral sequence and T cell responsiveness is performed at multiple time points in individual patients, one should not conclude that variant viral sequences have been selected by immune pressure.

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