The Cytotoxic T Lymphocyte Response to Multiple Hepatitis B Virus Polymerase Epitopes During and After Acute Viral Hepatitis

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Summary

Cytotoxic T lymphocytes (CTL) are thought to contribute to viral clearance and liver cell injury during hepatitis B virus (HBV) infection. Using a strategy involving the in vitro stimulation of peripheral blood mononuclear cells (PBMC) with HBV-derived synthetic peptides containing HLA-A2.1, -A31, and -Aw68 binding motifs, we have previously described CTL responses to several epitopes within the HBV nucleocapsid and envelope antigens in patients with acute hepatitis. In this study we define six HLA-A2–restricted CTL epitopes located in the highly conserved reverse transcriptase and RNase H domains of the viral polymerase protein, and we show that the CTL response to polymerase is polyclonal, multispecific, and mediated by $CD8^+$ T cells in patients with acute viral hepatitis, but that it is not detectable in patients with chronic HBV infection or uninfected healthy blood donors. Importantly, the peptide-activated CTL recognize target cells that express endogenously synthesized polymerase protein, suggesting that these peptides represent naturally processed viral epitopes. DNA sequence analysis of the viruses in patients who did not respond to peptide stimulation indicated that CTL nonresponsiveness was not due to infection by viral variants that differed in sequences from the synthetic peptides. CTL specific for one of the epitopes were unable to recognize several naturally occurring viral variants, except at high peptide concentration, underlining the HBV subtype specificity of this response. Furthermore, CTL responses against polymerase, core, and envelope epitopes were detectable for more than a year after complete clinical recovery and seroconversion, reflecting either the persistence of trace amounts of virus or the presence of long lived memory CTL in the absence of viral antigen. Finally, we demonstrated that wild type viral DNA and RNA can persist indefinitely, in trace quantities, in the serum and PBMC after complete clinical and serological recovery, despite a concomitant, vigorous, and sustained polyclonal CTL response. Since viral persistence is not due to escape from CTL recognition under these conditions, the data suggest that HBV may retreat into immunologically privileged sites from which it can seed the circulation and reach CTL-inaccessible tissues, thereby maintaining the CTL response in apparently cured individuals and, perhaps, prolonging the liver disease in patients with chronic hepatitis.

The hepatitis B virus (HBV)[1] is a noncytopathic DNA virus with a small, circular DNA genome that encodes several envelope (preS1, preS2, HBsAg), nucleocapsid (HBcAg, HBeAg), transactivating (X), and polymerase (pol) proteins and causes acute and chronic liver disease and hepatocellular carcinoma (1). It is thought that the immune response to one or more of these virus proteins is responsible for viral clearance and liver cell injury during HBV infection, and that HLA class I–restricted CTL play a major role in these events. In support of this hypothesis, we have recently shown that murine HBV envelope-specific CTL can cause severe acute liver disease when they are injected into

[1] *Abbreviations used in this paper:* ALT, alanine aminotransferase; B-LCL, B lymphoblastoid cell lines; HBcAg, HBeAg, HBsAg, hepatitis B core, envelope, or surface antigen, respectively; HBV, HCV, hepatitis B or C virus, respectively; pol, polymerase; Vpol, vaccinia virus construct encoding the HBV polymerase protein.

HBV-transgenic mice (2). We have also shown that the same CTL can also inhibit HBV gene expression noncytolytically in these animals (3), suggesting that virus inactivation can occur intracellularly without killing the infected cell. If the same events occur in the human liver during acute viral hepatitis, then the tissue-sparing effects of this aspect of the antiviral immune response could be substantial.

We have shown that patients with acute viral hepatitis mount a vigorous CTL response to multiple epitopes in the viral nucleocapsid (4–7) and envelope (8) proteins, whereas patients with chronic hepatitis and uninfected healthy individuals do not. Until now, however, the CTL response to the viral polymerase protein has not been defined. The HBV polymerase is a 93-kD protein that contains three functional domains that perform crucial functions in the viral life cycle: the terminal protein, reverse transcriptase, and RNase H (9). As such, it must be expressed very early during infection and, because it principally functions as an enzyme, it does not need to be expressed in very large amounts, unlike the structural proteins of the virus. Like other viral polymerases (10, 11), therefore, it would not be surprising if the HBV polymerase is a prominent target of the immune response, especially at the level of the CTL. More specifically, a CTL response against this protein could abort an incipient infection before it could spread. Indeed, this scenario is compatible with the well-known subclinical, self-limited outcome of most HBV infections in adults.

In this article, we demonstrate that acutely infected patients with clinically obvious viral hepatitis develop an HLA class I–restricted CTL response to multiple epitopes in the conserved functional domains of the HBV polymerase protein, while persistently infected patients with chronic hepatitis and normal uninfected controls do not. We also demonstrate that viral DNA can persist in the serum and PBMC, and that the CTL response to several polymerase, envelope, and nucleocapsid epitopes can persist for more than a year following complete clinical recovery and seroconversion. The data suggest that persistence of the CTL response after recovery could reflect either the continued presence of long-lived memory cells in the absence of antigen, or the persistence of trace amounts of wild-type virus in immunologically privileged compartments that can seed the peripheral blood with virus and maintain the CTL response long after clearance of viral antigens from the blood.

Materials and Methods

Patient Population. Nine HLA-A2$^+$ patients with acute hepatitis B, 9 HLA-A2$^+$ patients with chronic hepatitis B, and 10 HLA-A2$^+$ healthy, uninfected subjects were studied. The diagnosis of acute hepatitis B was based on clinical and biochemical evidence of acute liver injury according to standard diagnostic criteria, that is, jaundice and elevated values of alanine amino transferase (ALT) activity at least 20-fold greater than the upper limit of normal, together with serological evidence of acute HBV infection, that is, 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 δ or hepatitis C virus (HCV) infection (Abbott Laboratories, North Chicago, IL). Six of the nine patients recovered completely, with normalization of serum transaminases and clearance of HBsAg and HBeAg within 4 mo of initial diagnosis; the remaining three patients were lost to follow-up after their initial clinic visit. All patients with chronic hepatitis B were repeatedly serologically positive for HBsAg for >6 mo and displayed mildly to moderately elevated serum ALT activity. Normal controls had no clinical history of HBV infection and were serologically negative for all HBV markers. All subjects studied were repeatedly negative for antibodies to HCV and HIV. The study protocol was approved by the Human Subjects Committee at the Scripps Clinic and Research Foundation.

Synthetic Peptides, HBV Antigens, and Tetanus Toxoid. A panel of 9 and 10 residue peptides containing the HLA-A2.1 binding motif, that is, a leucine, methionine, or isoleucine in the second position and a valine, isoleucine, or leucine at the COOH terminus, were purchased from Chiron Mimotopes Peptide Systems (Clayton, Victoria, Australia). Lyophilized peptides were reconstituted at 20 mg/ml in DMSO (Malinckrodt Inc., Paris, KY) and diluted to 1 mg/ml with RPMI-1640 medium (Gibco Laboratories, Grand Island, NY). rHBcAg was obtained from bacterial extracts of *Escherichia coli* as previously described (12), and tetanus toxoid protein was purchased from Connaught Laboratories (Swiftwater, PA).

HLA-binding Affinity Analysis of Peptides. The HLA-A2.1 binding affinity of the peptides was determined by competitive binding studies with radiolabeled HBVcore18-27 peptide FLPSDYFPSV as previously described (13). Purified class I molecules were incubated at room temperature with various doses of the test peptides, together with 5–10 nM of the labeled peptide and 1 μM human β_2-microglobulin (Calbiochem Corp., La Jolla, CA). After 48 h, class I–peptide complexes were separated from free peptide by gel filtration. Class I–bound and free radioactivity was measured, and the doses of test peptides yielding 50% inhibition of the binding of the labeled peptide (IC50) were calculated.

Recombinant Expression Vectors. A recombinant vaccinia virus construct that encodes the HBV polymerase protein (Vpol) was produced by insertion of a 2766-bp fragment representing nucleotides 2290–1874 of the HBV genome (*ayw* subtype) into the SmaI site of the pSC11 vector by standard techniques as previously described (14). Stable transfectants that express HBpolAg were produced by transfection of EBV-transformed B lymphoblastoid cell lines (B-LCL) with a panel of EBV-based expression vectors that contain the corresponding coding regions of the HBV *ayw* subtype (15).

Stimulation of PBMC with Synthetic Peptides and rHBcAg. PBMC from patients and normal donors were separated on Ficoll-Histopaque density gradients (Sigma Chemical Co., St. Louis, MO), washed three times in HBSS (Gibco BRL Laboratories, Gaithersburg, MD), resuspended in RPMI-1640 (Gibco Laboratories) supplemented with L-glutamine (2 mM), gentamycin (10 μM), and 10% heat-inactivated human AB serum, and plated in a 24-well plate at 4 $\times$ 10^6 cells/well. rHBcAg (Biogen, Cambridge, MA) was added to the cell cultures at 1 μM and the synthetic peptides at 10 μM. In some of the experiments with healthy uninfected blood donors, rHBcAg was either omitted or replaced by 10 μM tetanus toxoid since these individuals have not been exposed to HBV previously and do not benefit from rHBcAg-induced T cell help. On days 3 and 10, 1 ml of RPMI 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, and they were tested for cytotoxic activity on day 14. Selected cultures that displayed peptide-specific cytolytic activity were separated into CD4$^+$ and CD8$^+$ populations by pan-

ning onto anti-CD4–coated flasks (Applied Immunosciences, Santa Clara, CA) and restimulated as described above.

Generation of HBV-specific CTL Lines and Clones. CTL lines were established as described above and enriched in highly cytotoxic $CD8^+$ CTLs by cloning at 10 and 3 cells/well in 96-well microtiter plates in the presence of 0.05 μM CD3-specific mAb (Coulter Immunology, Hialeah, FL), rIL-2 (100 U/ml), and 10^5 irradiated (3,000 rad) allogeneic PBMC. HBV-specific clones were established by cloning at 1 and 0.3 cells/well in the same way. Growing cultures were tested for cytotoxic activity against peptide-primed target cells on day 17, and cytotoxic lines and clones were expanded in 24-well plates and restimulated every 10 d.

Target Cell Lines. Autologous and allogeneic EBV-transformed B-LCL were either purchased from The American Society for Histocompatibility and Immunogenetics (Boston, MA) or established from our own pool of patients and normal donors as described (15). For most studies, JY cell targets (HLA-A2.1, B7, Cw7) were used. All target cells were maintained in RPMI with 10% (vol/vol) heat-inactivated FCS (GIBCO BRL).

Cytotoxicity Assay. Target cells consisted either of (*a*) allogeneic HLA-matched and mismatched B-LCL incubated overnight with synthetic peptides at 10 μM; (*b*) stable B-LCL transfectants as described above; or (*c*) B-LCL infected with recombinant vaccinia viruses. Vaccinia-infected targets were prepared by infection of 10^6 cells at 50 PFU/cell on a rocking plate at room temperature for 1 h followed by a single wash and overnight incubation at 37°C. Target cells were then 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. Stimulated PBMC from patients and normal controls were tested at E/T ratios of 50:1. All assays were performed in duplicate. Percent cytotoxicity was determined from the formula: 100 × [(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.

Flow Cytometry. 0.5–1.0 × 10^6 cells to be analyzed were washed once in PBS with 5% BSA and 0.02% sodium azide. The pelleted cells were then stained with a fluorescent probe–conjugated anti-CD4 and anti-CD8 mAB (Leu3a, Leu2a) and a similarly labeled control antibody (Becton Dickinson & Co., Mountain View, CA) for 30 min at 4°C. After three washes in PBS with 5% BSA and 0.02% sodium azide, cells were analyzed with a flow cytometer (FACScan®; Becton Dickinson & Co.).

HLA Typing. HLA typing of PBMC from patients and normal donors was performed by complement microcytotoxicity by use of HLA typing trays purchased form One Lambda (Los Angeles, CA).

Extraction of DNA and RNA from Serum and PBMC. HBV DNA was isolated from serum and PBMC by proteinase K digestion and phenol chloroform extraction exactly as previously described (8, 16). HBV RNA was extracted from PBMC samples that were positive for HBV DNA by use of the acid guanidinium thiocyanate-phenol-chloroform method exactly as previously described (16).

PCR. 10 μl of the DNA samples derived from sera or PBMC were amplified in a 100-μl reaction volume with reagents (GeneAmp; Perkin Elmer Cetus Instruments, Norwalk, CT). Pairs of oligonucleotide primers were synthesized on a DNA synthesizer (model 308B; Applied Biosystems, Inc., Foster City, CA). The direct PCR with the primer pair HBV246S (5′-GAGTCTAGACTCGTGGTGGA, position 246-265) and HBV1086AS (5′-GCGAGAAAGTGAAAGCCTG, position 1082-1100) was run in a programmable thermal cycler (Twin Block System; Ericomp, Inc., San Diego, CA) under the following conditions: 1 cycle of 5 min denaturation at 93°C, 2 min annealing at 56°C, 3 min elongation at 72°C, 35 cycles, in which the denaturation time was decreased to 30 s, the annealing time to 30 s and the elongation time to 1 min, followed by a 4-min elongation step at 72°C. For the nested PCR, 10 μl of the mixture resulting from the direct reaction was amplified with the primer pair HBV370S (5′-TATCGCTGGATGTGTCTGC, position 370-388) and HBVSIAN (5′-TTTAGAGAGTAACCCCATCT, position 851-870) under the same conditions. To sequence the part of the HBV genome containing the HBVcore18-27 epitope, selected samples were amplified with primers HBVXISE (5′-CAAGGTCTTACATAAGAGGA, position 1641-1660) and HBV2627AS (5′-CCTGGCAGGCATAATCAATT, position 2627-2646) in the direct PCR and with primers HBVX2SE (5′-CTCTTGGACTCTCAGCAATG, position 1661-1680) and HBVCBHINT (5′-TTGCCTGAGTGCAGTATGGTGAGG, position 2046-2069) in the nested PCR using the same PCR program. Extensive precautions were taken to eliminate sources of DNA contamination, as previously described (16).

Direct Sequencing of PCR Products. Direct sequencing of the PCR products was performed with the PCR product-sequencing kit (Sequenase™; Amersham/USB, Arlington Heights, IL) with the HBV-specific primers HBV370S, HBVSIAN, and CBH-INT. As shown by others (17), a viral subpopulation as low as 10% of the total population can be detected by direct sequencing of PCR products.

Reverse Transcriptase PCR. Approximately 1 μg of RNA derived from each PBMC preparation was used for cDNA synthesis. The reaction was performed with oligo dT primer for 1 h at 42°C as previously described with and without reverse transcriptase (16). HBV-specific cDNA sequences were amplified with PCR primers HBVXISE and HBV 1880AS (5′-GCACAGCTTGGAGGCTTGAA, position 1861-1880) and nested PCR primers HBVX2SE and HBV1860AS (5′-CAGTAGGACATGAACAAGAG, position 1841-1860) under the same PCR cycles described above.

Detection of Amplified DNA. 20-μl aliquots of the products from direct or nested PCR amplifications were analyzed by electrophoresis on a 0.9% agarose gel in the presence of 0.5 μM of ethidium bromide. DNA bands were visualized by UV fluorescence. For Southern blot analysis, the PCR-amplified DNA was transferred by capillary transfer to a nylon membrane (Hybond-N; Amersham Corp.) and hybridized with a HBV-specific DNA probe (^{32}P labeled by nick-translation) as previously described (18). The membranes were exposed to an X-ray film (XAR; Eastman Kodak Co., Rochester, NY) with an intensifying screen.

Results

Selection of Peptide Sequences Coded by the HBV Polymerase ORF. We screened the sequence of the HBV polymerase protein for the presence of 9-mers and 10-mers containing HLA-A2–specific binding motifs (19). This search yielded 220 candidate peptides. Out of this group, we selected 44 peptides because they were conserved in at least four of the seven HBV *adw* sequences present in the GenBank data base, and since the *adw* subtype of HBV is prevalent in our patient population. 15 of these peptides were selected for further analysis (Table 1) because they displayed an HLA-A2.1–binding affinity

Table 1. *Characteristics of Peptides Tested*

Peptide	Amino acid sequence	Frequency in HBV subtypes: Total (20)	*adw* (7)	*ayw* (5)	*adr* (7)	*ayr* (1)	HLA-A2.1-binding affinity (IC50 nM)	Acute HBV patients: Tested	responders
$Pol_{527-535}$	LLAQFTSAI	19	6	5	7	1	50	2	0
$Pol_{575-583}$*	FLLSLGIHL	19	6	5	7	1	10	9	6
$Pol_{816-824}$*	SLYADSPSV	8	4	4	0	0	14	9	3
$Pol_{502-510}$	KLHLYSHPI	19	6	5	7	1	17	3	0
$Pol_{655-663}$*	ALMPLYACI	19	6	5	7	1	25	5	2
$Pol_{551-559}$*	YMDDVVLGA	18	5	5	7	1	31	7	2
$Pol_{504-512}$	HLYSHPIIL	16	4	4	7	1	38	2	0
$Pol_{455-463}$*	GLSRYVARL	11	7	4	0	0	71	9	6
$Pol_{526-535}$	FLLAQFTSAI	19	6	5	7	1	70	2	0
$Pol_{149-158}$	YLHTLWKAGI	20	7	5	7	1	89	3	0
$Pol_{772-780}$	WILRGTSFV	16	6	5	4	1	278	4	0
$Pol_{773-782}$*	ILRGTSFVYV	16	6	5	4	1	313	7	3
$Pol_{765-774}$	LLGCAANWIL	16	6	5	4	1	357	2	0
$Pol_{424-432}$	NLSWLSLDV	18	5	5	7	1	385	3	0
Core18–27*	FLPSDFFPSV	9	5	4	0	0	3	9	4
Env335–343*	WLSLLVPFV	20	7	5	7	1	7	9	6

* Epitopes, that is, peptides to which at least one of the patients responded.

(corresponding to an IC50 of <500 nM), which we have recently shown to be a threshold below which most peptides are not immunogenic (20). 2 peptides that have previously been shown to be HLA-A2–restricted CTL epitopes (4, 8) derived from the core (HBc18-27) and envelope (HBs 335-343) proteins, were also included for the sake of comparison.

Analysis of the CTL Response to HBV Polymerase Peptides Containing the HLA-A2 Binding Motif. PBMC from 9 patients with acute hepatitis B were stimulated with three or more of the polymerase peptides. Two patients were tested serially for 18 mo during and after clinical recovery from acute hepatitis and complete seroconversion. For comparison, we also tested 9 HLA-A2$^+$ patients with chronic hepatitis B and 10 healthy, uninfected control subjects.

Eight of the nine acutely infected patients responded to at least one of the polymerase peptides and, as can be seen in Fig. 1, six of the peptides were recognized by at least one patient. These results demonstrate the polyclonality and multispecificity of the CTL response against the polymerase protein during acute viral hepatitis, similar to what we observed for core- and envelope-specific CTL responses. Indeed, seven of these patients also recognized one or both of the two control peptides HBV core18-27 and HBV env335-343, while one patient (A-5) recognized only HBV env335-343. Only one of the acutely infected patients failed to respond to all of the peptides in this panel.

In contrast, none of the uninfected controls responded to any of the peptides used in this study (Fig. 1), suggesting that the CTL responses observed in the acutely infected patients represented in vitro secondary responses resulting from exposure to infected cells in vivo. Furthermore, none of the nine patients with chronic hepatitis produced a response.

As shown in Table 1, the HLA-binding affinity for four of these six peptides (marked with an asterisk) is high, that is, the IC50 is <50 nM, suggesting a direct relationship between HLA-binding affinity and immunogenicity even among this group of relatively high-affinity peptides.

It is important to note that we may have underestimated the number of CTL epitopes in our panel of peptides because several peptides were tested in only two to three patients due to limited quantities of PBMC in several patients. Accordingly, when limited numbers of PBMC were available, polymerase peptides that were shown to elicit a response in previously tested patients were preferentially used.

$CD8^+$ CTL Lines and Clones Specific for $Pol_{455-463}$, $Pol_{575-583}$, and $Pol_{816-824}$ Recognize Endogenously Synthesized Polymerase Antigen. Two HLA-A2$^+$ patients (A-1 and A-2) with acute hepatitis who responded particularly strongly to $Pol_{575-583}$, $Pol_{455-463}$, and $Pol_{816-824}$ (Fig. 1) were chosen for further analysis. After 2 wk of in vitro stimulation, selected cultures that displayed peptide-specific CTL responses were enriched for CD4$^+$ and CD8$^+$ subsets by panning, restimulated in vitro with peptide and finally tested for recognition of endogenously processed polymerase antigen. As shown in Fig.

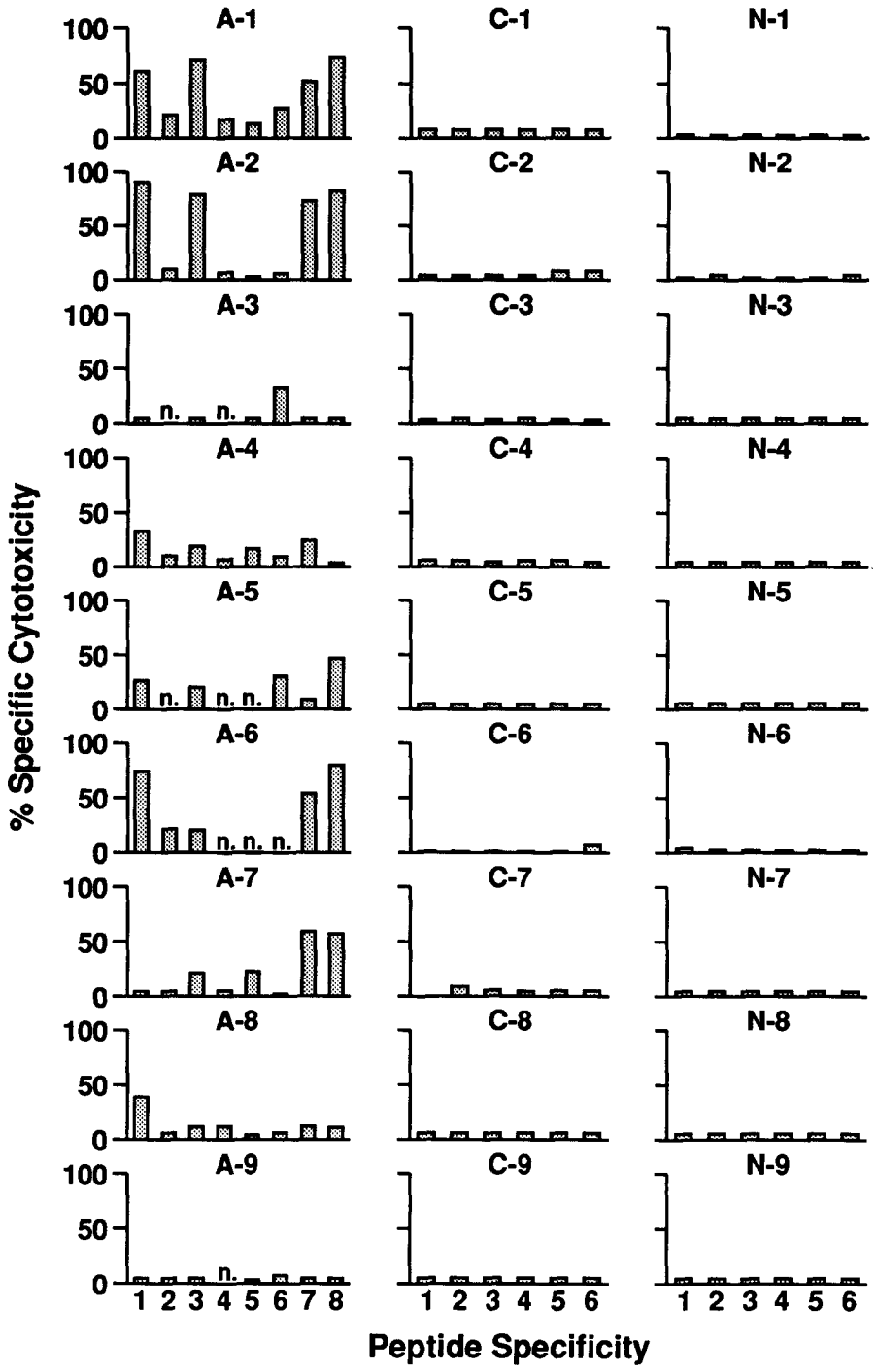

Figure 1. HBV specific CTL response in patients with acute hepatitis (A-1–A-9), chronic hepatitis (C-1–C-9), and normal subjects (N-1–N-9). PBMC were stimulated with 10 μM of peptide for 2 wk as described in Materials and Methods and tested in a 4-h ^{51}Cr release assay against JY target cells prepulsed 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 percentage of specific lysis in a 4-h ^{51}Cr release assay at an E/T ratio of 50:1. The peptide specificity is as follows: 1, Pol455-463; 2, Pol551-559; 3, Pol 575-583; 4, Pol655-663; 5, Pol773-782; 6, Pol816-824; 7, Core18-27; 8, Env335-343 (see Table 1 for the characteristics of these peptides). *n*, not tested.

2, only the CD8$^+$ fraction of each cell line recognized target cells either pulsed with peptide or stably transfected with the polymerase expression vector. These results suggest that these three peptides represent native epitopes produced by the cellular processing of the endogenously synthesized polymerase protein and recognized in the context of class I HLA molecules by CD8$^+$ CTL.

To obtain pure CD8$^+$ cell lines and further characterize these T cell responses, each of the three responding cell lines was cloned by limiting dilution in the presence of anti-CD3, irradiated allogeneic PBL, and IL-2. All of the derivative cytotoxic lines were highly enriched in CD8$^+$ cells as determined by FACS® analysis (Fig. 3). Furthermore, five of the six Pol$_{455-463}$–specific CTL clones derived in this manner also consisted of CD8$^+$ cells, except for one clone from patient A-2 (clone 48) that was found to be CD4$^+$. Unfortunately, this clone was lost before additional studies were possible (Fig. 3).

Four highly cytotoxic long-term CTL lines and two clones specific for Pol$_{455-463}$-pulsed targets were chosen for further

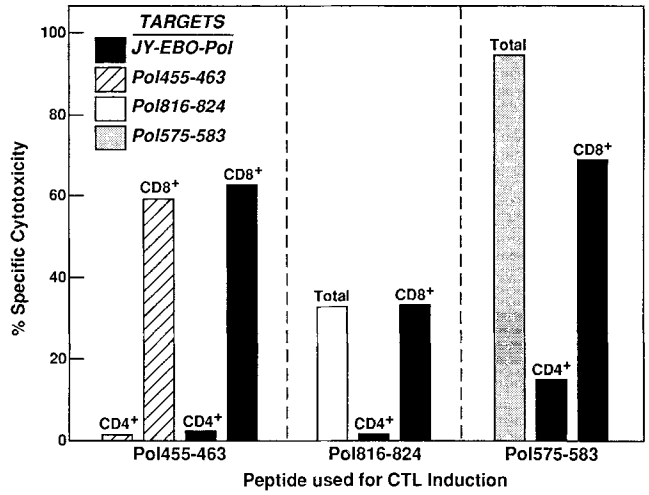

Figure 2. CD8+ cells recognize endogenously synthesized antigen in target cells sharing the HLA-A2 allele. Epitope-specific lines were generated by stimulating PBMC from patient A-1 with 10 μM of the individual peptide for 3 wk, with weekly restimulation as described in Materials and Methods. On day 15, CD4+ (positive selection) and CD8+ (negative selection) -enriched lines were generated from the original bulk culture by panning. FACS® analysis showed an average enrichment of the selected subset by a factor of 3. Results shown represent percentage of specific lysis in a 4-h ^{51}Cr release assay at an E/T ratio of 30:1. Targets (JY-EBV) were either pulsed with 10 μM of the corresponding peptide overnight or stably transfected with the origin for plasmid replication (EBO) polymerase expression vector.

analysis (Table 2 and Fig. 3). The quality of the cytotoxic activity was assessed by varying both the amount of peptide used to pulse the target cells and by the E/T ratios. Target cells pulsed with as little as 0.01 μM of peptide (Table 2) were efficiently lysed. Both peptide pulsed and Vpol-infected target cells at E/T ratios as low as 1.6:1 (Fig. 3) were also efficiently lysed, confirming the ability of these CTL to recognize endogenously synthesized antigen. Control target cells pulsed with no peptide (Table 2) or with an irrelevant peptide (Table 3), which is an HLA-A2–restricted epitope in HCV-infected patients (21), were not lysed, nor were cells infected by Wt-vaccinia virus used as control.

HLA Restriction Analysis and Fine Specificity of HBV Polymerase–specific Lines and Clones. To identify the restric-

Table 2. *Recognition of GLSRYVARL-pulsed JY-EBV by CTL Is Peptide Dose Dependent*

		Peptide concentration*				
Patient	Line	10	1	0.1	0.01	0 (μM)
		(% cytotoxicity)				
A-1	67–68	41	26	19	13	5
A-2	10	75	56	52	25	12
A-2	30	69	40	40	19	6

* JY target cells were pulsed with GLSRYVARL at the indicated concentrations to generate target cells as described in the text.

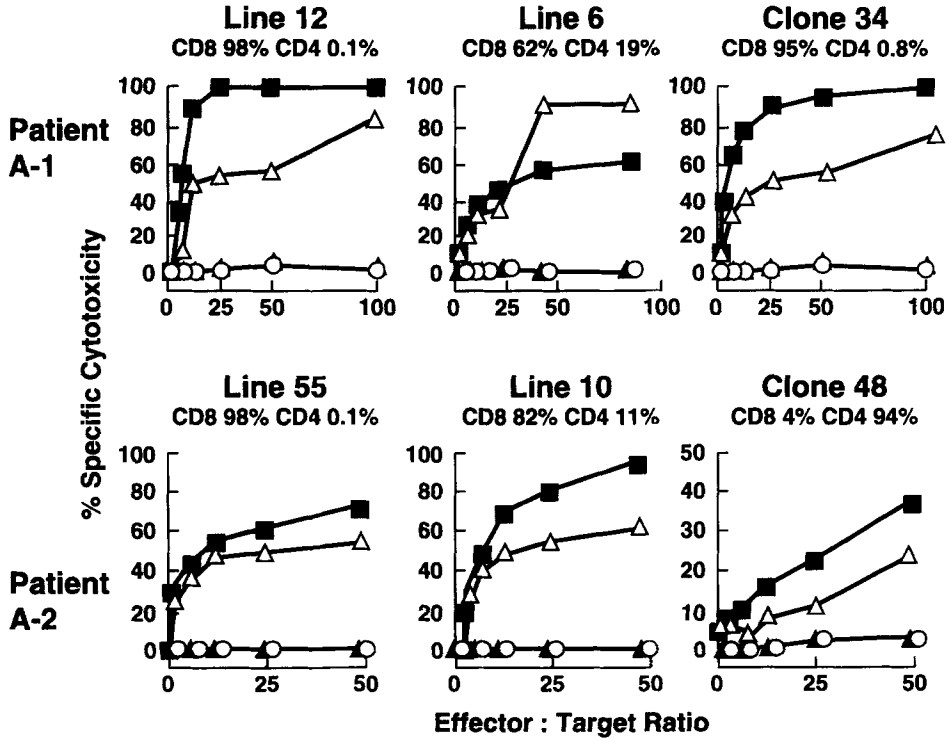

Figure 3. CTL response to $Pol_{455-463}$ GLSRYVARL. Epitope-specific lines and clones, generated by stimulation of PBMC from patients A-1 and A-2 with $Pol_{455-463}$ peptide, were generated as described in Materials and Methods and tested at varying E/T ratios against target cells (JY-EBV), pulsed with 10 μM of the corresponding peptide (■) overnight or infected with recombinant Vpol (△), in a standard 4-h ^{51}Cr release assay. Wild-type vaccinia virus (▲) or JY-EBV peptides without peptide (○) were used as a control.

Table 3. *Induction of CTL with $Pol_{455-463}$-variant Peptides*

Inducing peptide*	Target peptide‡				
	GLSRYVARL	GLPRYVARL	SGLSRYVARL	GLSRYVARLS	KLVALGINAV§
			(% *specific cytotoxicity*)		
GLSRYVARL	54	18	40	41	2
GLPRYVARL	1	0	1	0	
SGLSRYVARL	0	0	0	0	
GLSRYVARLS	1	1	0	0	

* $Pol_{455-463}$-variant peptides used to stimulate PBMC.
‡ JY target cells pulsed with indicated peptides at 10 μM.
§ This peptide, derived from the HCV NS3 protein, is recognized by HLA-A2–restricted CTL from HCV-infected patients (21).

tion element used by the HBV $Pol_{455-463}$–specific CTL, cytotoxic lines and clones from patients A-1 and A-2 were tested against allogeneic EBV-B cell lines sharing individual HLA class I alleles with the effector cells. As shown in Fig. 4, not only is HLA-A2 the sole class I allele shared by these two patients, but their CTL only lyse peptide pulsed target cells that share this allele. We conclude that $Pol_{455-463}$-specific CTL from both patients are HLA-A2 restricted.

Peptides containing COOH- and NH_2-terminal truncations and elongations of the $Pol_{455-463}$ sequence (GLSRYVARL) were synthesized to determine the optimal length and precise termini of this epitope. As shown in Fig. 5 *A*, truncation of glycine 455 or leucine 463 greatly reduced the HLA-A2–binding affinity of the peptides and totally abrogated their recognition by CTL induced by the original peptide $Pol_{455-463}$. Interestingly, elongation of the peptide by adding a single serine residue normally present upstream of the NH_2 terminus or downstream of the COOH terminus of $Pol_{455-463}$ did not diminish its recognition by CTL (Fig. 5 *A*) and may have even increased recognition, despite the fact that the HLA-A2–binding affinity of the extended peptides was reduced 4–10-fold relative to the original peptide (Fig. 5 *A*). Despite this, the extended peptides were unable to induce a CTL response in a patient who responded quite vigorously to the $Pol_{455-463}$ sequence (Table 3). Collectively,

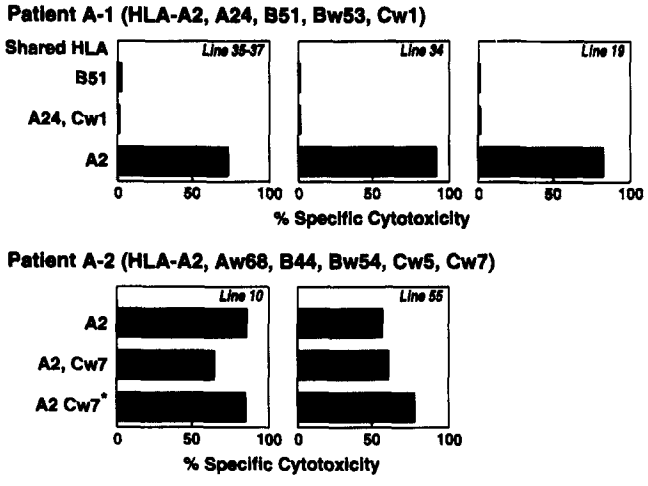

Figure 4. HLA restriction of epitope $Pol_{455-463}$. $Pol_{455-463}$-specific lines from patients A-1 and A-2, generated by stimulation with 10 μM of $Pol_{455-463}$ peptide, were tested against allogeneic, partly HLA-matched EBV-B cells prepulsed overnight with 10 μM of the same peptide. Sharing HLA class I at other loci did not render target cells susceptible to lysis. Cytotoxicity was measured at an E/T ratio of 50:1 in a 4-h ^{51}Cr release assay. The asterisk marks an EBV-B cell line from a separate donor.

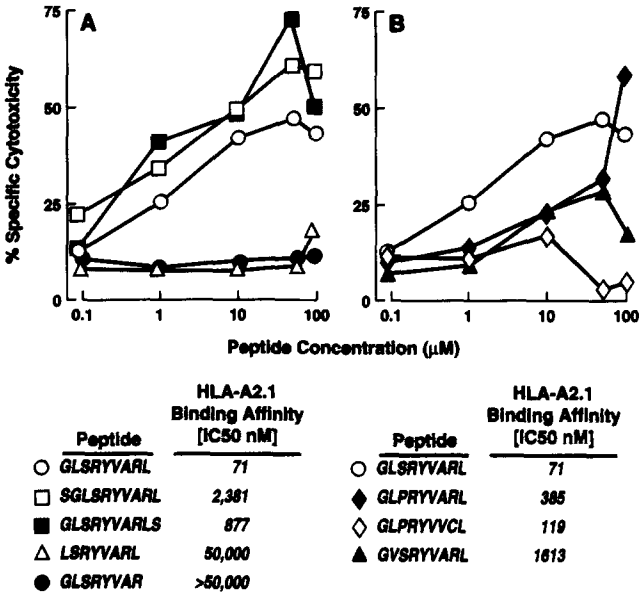

Figure 5. Recognition of truncated or elongated (*A*) and variant (*B*) peptides by $Pol_{455-463}$-specific CTL lines, generated by weekly stimulation of PBMC from patient A-1 with peptide $Pol_{455-463}$ for 4 wk. Cytotoxicity was measured at an E/T ratio of 50:1 in a 4-h ^{51}Cr release assay against JY-EBV cells prepulsed with varying amounts of the same peptide overnight. The sequence GLSRYVARL is present in 7/7 HBV subtype *adw* sequences, 4/5 *ayw*, 0/7 *adr*, and 0/1 *ayr* sequences, GLPRYVARL is present in 6/7 *adr* and 1/1 *ayr* sequences, GLPRYVVCL is present in 1/7 *adr*, and GVSRYVARL is present in 1/5 *ayw* sequences. The HLA-A2.1–binding affinity is calculated as the amount of test peptide needed to inhibit the binding of the radiolabeled HBVcore 18-27 (FLPSDYFPSV) peptide by 50% (IC50).

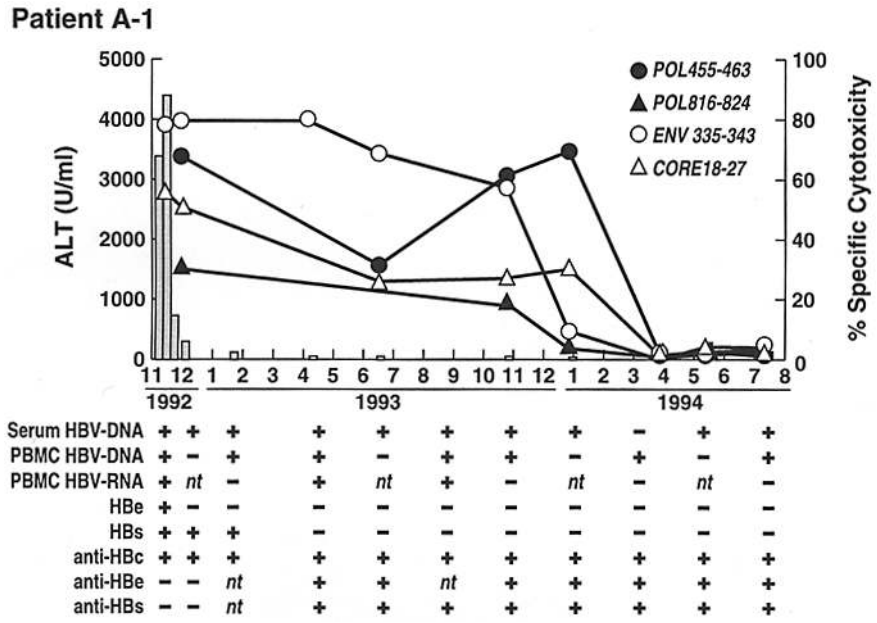

Figure 6. Time course of CTL response after acute hepatitis B (patient A-1). At each time point, PBMC were stimulated with individual peptides for 2 wk and cytotoxicity was measured at an E/T ratio of 50:1 in a 4-h ^{51}Cr release assay against JY-EBV cells prepulsed overnight with 10 μM of the same peptide. Serological and virological analysis was performed as described in Materials and Methods. Serum ALT activity is shown as bars. At each time point shown after December 1992, transaminase levels were found to be normal.

these data strongly suggest that the GLSRYVARL sequence in $Pol_{455-463}$ represents the minimal optimal epitope recognized by these CTL. If one assumes that the density of the extended peptides at the surface of APC is lower than the density of the shorter peptide, due to differences in HLA-A2–binding affinity, perhaps the inability of the extended peptides to induce a CTL response, while they are quite capable of being recognized by preexisting CTL at the same peptide concentration, reflects the fact that higher determinant densities are required for CTL induction than for recognition (22).

HBV Subtype Specificity of Cytotoxic T Cell Clones Recognizing $Pol_{455-463}$. The GLSRYVARL sequence is present in 7/7 and 4/5 HBV *adw* and *ayw* subtype sequences, respectively, present in the GenBank data base. The amino acid sequence of the remaining *ayw* isolate in the data base is GVSRYVARL. The sequence of 6/7 *adr* and 1/1 *ayr* isolates is GLPRYVARL and the sequence of the remaining *adr* isolate is GLPRYVVCL. Direct sequencing of the viral DNA amplified from the serum of five of the nine patients (A-1, A-2, A-6, A-7, and A-9) with acute hepatitis B by nested PCR demonstrated that the deduced HBV amino acid sequence was identical to GLSRYVARL in these patients. Interestingly, two of those patients (A-7 and A-9) did not mount a CTL response to GLSRYVARL. These results suggest that the failure of patients A-7 and A-9 to respond to this epitope is not due to viral variation within the epitope. HBV DNA sequence analysis could not be performed in the other four patients because two of them had already cleared the HBV DNA at the time of presentation, and serum was not obtained from the other two.

Peptides containing the sequences of the different viral subtypes were tested for recognition by GLSRYVARL-stimulated PBMC to assess the possible cross-reactivity of the CTL response. All of the variants were less efficiently recognized than the prototype sequence against which the CTL were raised. Most notably, GLPRYVVCL was not recognized at all, even at very high peptide concentration, despite the fact that its HLA-A2.1–binding affinity is similar to that of the prototype peptide GLSRYVARL (Fig. 5). This suggests that serine 457, alanine 461, and arginine 462 may represent TCR contact sites (epitope residues) in this peptide.

The GVSRYVARL variant, which contained a single substitution at leucine 456, a presumptive HLA contact site (agretope residue), was very poorly recognized by the CTL, commensurate with the ninefold reduction in its HLA-A2–binding affinity. Interestingly, however, the NH_2 and COOH terminally extended peptides described above were very well recognized by the CTL despite the fact that they displayed comparably reduced HLA-A2–binding affinities (Fig. 5 *A*). These observations suggest that leucine 456 not only serves as an agretope residue, but it may also influence the TCR-binding affinity of the peptide, perhaps by exerting conformational influences on serine 457, the adjacent putative TCR contact site.

HBV DNA Can Persist in the Serum and PBMC Long After Complete Recovery from Acute Viral Hepatitis Despite a Vigorous Concurrent Polyclonal HBV-specific CTL Response. In serial studies of two patients, we demonstrated that the CTL response to multiple HBV epitopes begins very early and can persist for an extended period after complete clinical and serological recovery. Both patients A-1 (Fig. 6) and A-2 (Fig. 7) recovered from acute hepatitis B within a month after onset of disease as determined by normalization of liver enzymes. Serological recovery was evident by the prompt loss of HBsAg and HBeAg and the development of the corresponding antibodies between 1 and 6 mo after disease onset. However, more in-depth molecular and immunological analysis revealed major differences between these two patients.

For example, patient A-1 failed to clear HBV DNA from his serum and HBV DNA and RNA from his PBMC for

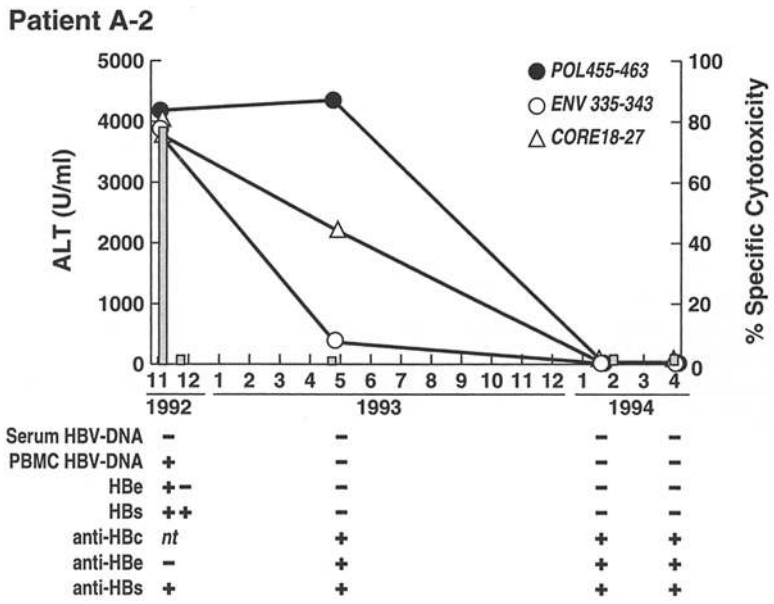

Figure 7. Time course of CTL response after acute hepatitis B (patient A-2). At each time point, PBMC were stimulated with individual peptides for 2 wk and cytotoxicity was measured at an E/T ratio of 50:1 in a 4-h ^{51}Cr release assay against JY-EBV cells prepulsed overnight with 10 μM of the same peptide. Serological and virological analysis was performed as described in Materials and Methods. Serum ALT activity is shown as bars. At each time point after the initial one in November 1992, transaminase levels were found to be normal.

up to 18 mo after resolution of his liver disease, despite the fact that he displayed a vigorous, polyclonal CTL response to four different CTL epitopes in the polymerase, envelope, and nucleocapsid proteins (Fig. 6) during the first 12 mo of his recovery. Eventually, the CTL response waned in this patient despite the persistence of viral DNA in his serum. Importantly, the deduced amino acid sequence for these three epitopes ($Pol_{455-463}$, $Env_{335-343}$, and $Core_{18-27}$) was identical in early and late samples, suggesting that viral persistence was not due to the selection of mutant viral genomes by the strong CTL response in this patient, and that the waning of the response was not due to the failure of the virus to encode the corresponding epitopes.

In contrast to patient A-1, all viral markers cleared very rapidly in patient A-2. This patient also displayed a vigorous, polyclonal CTL response to the same antigens early in the course of his infection that persisted for >6 mo after recovery and eventually waned (Fig. 7). These results illustrate that HBV-specific CTL, presumably memory T cells, remain in the circulation for an extended but finite time period after all traces of virus have disappeared from peripheral blood.

Discussion

We have previously demonstrated that patients with acute HBV infection develop HLA class I–restricted CTL responses to the viral nucleocapsid and envelope epitopes by stimulating their PBMC with sets of long (15–20 mers) overlapping synthetic peptides. This strategy was inherently rather inefficient, and it was necessary to screen very large numbers of peptides in many patients in order to identify a small number of epitopes (8). In each case, the inducing peptides were shown to contain shorter (9–11 residues) subunits that actually represented the minimal, optimal epitopes recognized by the CTL, and these subunits were shown to contain allele-specific binding motifs for HLA-A2 (6, 8), HLA-A31 (7), or HLA-Aw68 (7).

Based on these observations, we subsequently improved the efficiency of this technique by starting with peptides that contain specific HLA-binding motifs and limiting the analysis to subjects who express the corresponding allele (8). In the current study, we further refined this strategy by selecting peptides based on two additional parameters: high sequence conservation and high HLA-binding affinity. The first parameter increases the likelihood that the subject will have been primed to the corresponding epitope in vivo, especially if one is studying the CTL response to viruses with high mutation rates, such as HBV. The second parameter is based on the recently demonstrated direct correlation between immunogenicity and HLA-binding affinity (20).

We now report the successful use of this new strategy to efficiently study the HLA-A2–restricted CTL response to the HBV polymerase protein in patients with acute viral hepatitis. We started by scanning the aligned amino acid sequences of 20 HBV genomes whose DNA sequence is contained in the GenBank data base for peptides containing the HLA-A2.1–binding motif (i.e., 9-mers and 10-mers containing leucine, methionine, or isoleucine in position 2 and valine, isoleucine, or leucine at the COOH terminus), using a custom computer program we developed for this purpose. This search of the 833-residue polymerase protein revealed 220 candidate peptides for CTL analysis.

Given the limited amount of blood that can be ethically obtained from an acutely infected patient and the large number of lymphocytes (4×10^6) that are used to screen each peptide (8), only a handful of peptides can be analyzed in each patient. For this reason, we limited our analysis to those peptides that were highly conserved among the seven HBV isolates of the *adw* subtype present in our data base, since this subtype is prevalent in our patient population. 44 peptides

that were found to be present in at least four of the seven HBV *adw* isolates were then analyzed for their HLA-binding affinity via a direct binding assay. 15 of these 44 peptides displayed HLA-A2.1-binding affinities of <500 nM. Based on the results of a concurrent study in which we demonstrated that an affinity threshold of ~500 nM appears to be associated with the capacity of peptide epitopes to elicit CTL responses (20), these 15 peptides were selected for further analysis.

6 of the 15 polymerase peptides studied (40%) were found to be capable of eliciting a CTL response in patients with acute viral hepatitis (Table 1), and the CTL response to three of these epitopes was characterized in greater detail. Based on the success of this strategy, we believe that it will be generally useful for the analysis of the CTL response to many different antigens.

Several important aspects of this study deserve emphasis. First, the peptide-based in vitro stimulation protocols used detect HBV polymerase-specific CTL that were primed in vivo, since we observed CTL responses only in acutely infected patients and not in uninfected controls (Fig. 1). This is consistent with our earlier studies of the peptide-activated CTL response to the HBV nucleocapsid (5, 7) and envelope (8) antigens. Furthermore, the $Pol_{455-463}$-specific CTL are effective at low (1.6:1) E/T cell ratios (Fig. 3), and they kill targets pulsed with low (10 nM) peptide concentrations (Table 2), compatible with a physiological role for this response in vivo.

Second, the $Pol_{455-463}$-, $Pol_{575-583}$-, and $Pol_{816-824}$-specific CTL responses are principally mediated by $CD8^+$ T cells (Figs. 2 and 3), and the $Pol_{455-463}$-specific response in patients A-1 and A-2 was formally shown to be HLA-A2.1 restricted (Fig. 4), also in keeping with our previous studies (4–7). In fact, the responses in most patients appear also to be HLA-A2.1 restricted, since this is the only class I allele that they share with the JY target cell line (HLA-A2.1, B7, Cw7) we used in our study. It is interesting that one of the $Pol_{455-463}$-specific CTL clones derived from patient A2 was $CD4^+$. This peptide also contains HLA DR1- (23), DR4- (24), DR7- (25), DR11- (26), and DQ3.1- (27) binding motifs which might be able to induce a class II-restricted response in this patient whose class II phenotype is DR0101, DR1104, DQ0501, DQ0301. Unfortunately, this clone was lost before it could be analyzed further.

Third, $Pol_{455-463}$, $Pol_{575-583}$ and $Pol_{816-824}$ epitopes appear to represent naturally processed viral epitopes since peptide-activated CTL recognized endogenously synthesized polymerase antigen expressed by target cells, either stably transfected with a polymerase-EBV construct (Fig. 2) or infected with a polymerase-encoding vaccinia virus (Fig. 3). Thus, CTL specific for these epitopes should be able to recognize infected hepatocytes, and therefore could play a role in viral clearance and liver cell injury in vivo.

Fourth, as observed in other viral infections (10, 11), the HBV polymerase appears to be quite immunogenic at the CTL level, since it is present in infected cells in trace quantities compared with structural proteins but induces an equivalent CTL response. It is interesting to note that the polymerase is rapidly degraded from its COOH terminus, where most of the epitopes we identified in this study are located, perhaps facilitating efficient processing of epitopes located in this region of the protein. Since the polymerase enzyme is required for the earliest steps in the viral life cycle, polymerase-specific CTL may play an important role in limiting viral spread and may thereby attenuate disease severity. Perhaps the fact that most individuals who are infected by HBV develop a relatively mild, often subclinical, transient infection is due in part to the CTL response to this early antigen. It will be necessary to examine patients during the incubation phase of HBV infection to determine if there is a temporal hierarchy in the timing of the CTL response to each of the viral antigens.

Fifth, unlike the vigorous polymerase-specific CTL response we observed in acutely infected patients who cleared the virus, we were unable to detect a response in patients with chronic HBV infection (Fig. 1). This is consistent with our previous observations relating to nucleocapsid- and envelope-specific CTL responses (5, 8). It has to be noted, however, that HBV envelope-specific CTL have been detected in the intrahepatic lymphomononuclear cell infiltrate in patients with chronic hepatitis (27), but not in the peripheral blood. Therefore, while the CTL response is weaker in such patients relative to patients with acute hepatitis, it is not completely absent. Thus, a vigorous CTL response to these antigens may lead to viral clearance, perhaps by destruction of infected hepatocytes. A weak response could cause an indolent necroinflammatory liver disease by destroying some infected cells but leaving others to produce new virus and maintain the infection. We have recently reported, in a transgenic mouse model system, that class I-restricted CTL can noncytolytically downregulate HBV gene expression when they are activated by antigen recognition in vivo, and we have shown that they do so by an IFN-γ-dependent mechanism (3).

Sixth, in studies of truncated and extended homologues of one of the polymerase epitopes (GLSRYVARL), we demonstrated that this peptide, which was selected for analysis based on the HLA-A2-binding motif, actually represents the minimal, optimal epitope sequence recognized by these CTL (Fig. 5 *A*). This confirms our earlier studies with long peptides containing an internal HLA-A2-binding motif to induce a CTL response in which we demonstrated that optimal CTL recognition and induction was achieved with truncated peptides that precisely matched the predicted binding motif (4–7).

Although the GLSRYVARL epitope is present in all seven cloned HBV *adw* isolates and 4/5 *ayw* isolates in the GenBank data base, it is not absolutely conserved. As shown in Fig. 5, serine 457 is replaced by a proline (GLPRYVARL) in 6/7 *adr* and 1/1 *ayr* isolates, while additional substitutions are present in 1/7 *adr* (GLPRYVVCL) and 1/5 *ayw* (GVSRYVARL) isolates. As shown in Fig. 5 *B*, none of the substituted peptides was recognized as well as the inducing peptide by GLSRYVARL-specific CTL. GLPRYVARL also failed to induce CTL in contrast to GLSRYVARL. The fact that GLPRYVVCL was not recognized at all, despite the fact that it bound to HLA-A2 with an affinity similar to that

of the prototype peptide, suggests that serine 457, alanine 461, and or arginine 462 might represent TCR contact sites (epitope residues) in GLSRYVARL. Indeed, the variant peptide containing a proline for serine substitution at position 457 (GLPRYVARL) is recognized very poorly despite an HLA-A2-binding affinity comparable to that of the prototype peptide. It is interesting to note that the variant peptide containing a valine for leucine 456 substitution at anchor position 2 in the peptide was very poorly recognized by the CTL (Fig. 5 *B*) despite a relatively good HLA-binding affinity. It is possible that position 456 may influence not only the HLA-A2-binding affinity of the peptide, but it may also affect its TCR-binding affinity, perhaps by influencing the accessibility of the adjacent serine 457 to the TCR.

Finally, serial prospective analysis of the clinical, serological, virological, and immunological responses to acute HBV infection in two patients (Figs. 6 and 7) revealed the existence of distinct response patterns and illustrated certain aspects of HBV immunobiology that had not been demonstrated previously. The CTL response was detectable very early in the disease process in both individuals, and it was vigorous, polyclonal, and multispecific, as we have previously reported (4, 5, 8). Interestingly, the CTL response lasted for several months after complete clinical and serological recovery in both patients (at least 6 mo in patient A-2 and 12–15 mo in patient A-1). This probably reflects the persistence of memory CTL in the peripheral blood in patient A-2, who displayed no traces of HBV very soon after disease onset (Fig. 7), but in the case of patient A-1, who has not cleared HBV DNA from his serum >18 mo after recovery (Fig. 6), it could also reflect the ongoing CTL activation events.

While this remains speculative at present, the very prolonged CTL response in patient A-1, coincident with the continuing persistence of HBV DNA in the serum and the persistence of HBV RNA in the PBMC for several months after seroconversion, suggests that the CTL response could have been maintained in this patient by continued antigenic stimulation by virus that the CTL could not eradicate. Since DNA sequence analysis revealed that epitope mutations did not account for viral persistence in this patient, it is possible that the virus could have been seeded into the circulation from infected cells located in immunologically privileged sites that, unlike hepatocytes, are not accessible to the CTL, for example, renal tubular epithelial cells, as has been recently demonstrated in a transgenic mouse model (28). While this event could explain sustained CTL response without virus eradication, it does not explain why the CTL response eventually waned in this patient. The CTL response detected could be merely reflective of the long half-life of CTL maintained by continuous viral stimulation. Alternatively, the waning response could reflect CTL exhaustion in view of the sustained high-level CTL activation in this patient.

Whatever proves to be the correct explanation for these interesting observations, this study demonstrates for the first time that HBV has the potential to persist indefinitely, albeit in trace quantities, in some patients who have otherwise completely recovered from acute viral hepatitis, despite the coexistence of a strong, polyclonal, and multispecific CTL response. This confirms our earlier report of prolonged viral DNA persistence after acute hepatitis in patients whose CTL response was not examined (16). This represents a new category of persistent HBV infection that forms a link in the spectrum between classic acute hepatitis with complete viral clearance and classic chronic hepatitis with persistent high-level hepatic infection.

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