

# Cytotoxic T Lymphocyte Responsiveness after Resolution of Chronic Hepatitis B Virus Infection

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## Abstract

Clearance of the hepatitis B virus (HBV) during acute hepatitis is associated with a strong, polyclonal, multispecific cytotoxic T lymphocyte (CTL) response to the viral envelope, nucleocapsid and polymerase proteins that persists for decades after clinical recovery. In contrast, chronically infected patients usually fail to mount a strong CTL response to this virus. In this study we demonstrate that chronically infected patients who experience a spontaneous or interferon-induced remission develop a CTL response to HBV that is similar in strength and specificity to patients who have recovered from acute hepatitis. The results suggest that specific immunotherapeutic enhancement of the CTL response to HBV should be possible in chronically infected patients, and that it could lead to viral clearance in these individuals with resolution of chronic liver disease. (*J. Clin. Invest.* 1996. 97:1655–1665.) Key words: HBV • CTL • interferon • HLA-A2 • limiting dilution analysis

## Introduction

The hepatitis B virus (HBV)<sup>1</sup> is a noncytopathic DNA virus that causes acute and chronic hepatitis and hepatocellular carcinoma (1). Patients who successfully clear acute HBV infection characteristically mount a strong, polyclonal and multispecific cytotoxic T lymphocyte (CTL) response to HBV (2-6) that lasts for decades following recovery, which is probably maintained by traces of virus that persist indefinitely after clinical and serological recovery (Rehermann, B., C. Ferrari, and F. V. Chisari, manuscript submitted for publication). Approximately 5% of adults and 95% of neonates who become infected by HBV develop persistent HBV infection (7), which now affects more than 300 million people worldwide (8). HBV-specific CTL are usually not detectable in the peripheral

blood of these patients, but they are detectable at low precursor frequency at the site of inflammation, the liver (9, 10). Hence, it appears that HBV-specific CTL could contribute to chronic inflammatory liver disease by destroying some of the infected hepatocytes, but their precursor frequency is probably too low to eliminate all of the infected hepatocytes and mediate viral clearance.

It is noteworthy, however, that 5–10% of chronically infected patients clear HBV DNA and hepatitis B envelope antigen (HBeAg) spontaneously each year, and this can be followed by clearance of hepatitis B surface antigen (HBsAg) (11, 12). Importantly, spontaneous clearance of HBV-DNA and HBeAg is generally preceded by an exacerbation of liver disease activity (13) and accompanied by increased CD4<sup>+</sup> proliferative T cell responses to HBV (13, 14), suggesting that latent, immune-mediated clearance mechanisms can become spontaneously activated in some individuals. Furthermore, interferon alpha treatment substantially increases the annual rate of HBeAg and HBsAg clearance, with the same antecedent flare in disease activity seen during spontaneous clearance episodes (15). These observations support the notion that HBV-specific CTL are present in chronically infected patients but that they are quantitatively or qualitatively ineffective with respect to HBV clearance. Most importantly, they also imply that this defect is reversible, at least in some individuals.

To test this hypothesis, in the current study we analyzed the HBV-specific CTL response in a group of patients who had previously been treated with interferon alpha for chronic HBV infection, some of whom had cleared the virus while others had not. We also studied a group of untreated patients with chronic hepatitis, some of whom had previously experienced a spontaneous remission, and a group of acutely infected patients who served as a positive control. We now report that HBV-specific CTL are readily detectable in the peripheral blood in many patients who successfully terminate chronic hepatitis.

## Methods

**Patient population.** 26 patients with chronic hepatitis B, 2 with acute hepatitis B, and 12 uninfected healthy control subjects were studied (Table I). All 40 were positive for HLA-A2 by microcytotoxicity assay.

The 26 patients with chronic hepatitis B were classified into five groups (Table II): (a) eight patients who had never been treated with alpha IFN were positive for HBsAg, HBeAg, and HBV-DNA (untreated patients UT 1–8); (b) five patients who received IFN and had a long-term beneficial response having cleared both HBeAg and HBsAg (IFN complete responders: I-CR 1–5); (c) five patients who received IFN and had a partial beneficial response having cleared HBeAg but not HBsAg (IFN partial responders: I-PR 6–10); (d) four patients who received IFN and did not have a sustained response (IFN nonresponders: I-NR 11–14) remaining positive for HBsAg and HBeAg or displaying elevated aminotransferases; (e) four patients who had not been treated with alpha IFN but had spontaneously cleared HBeAg

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1. Abbreviations used in this paper: CRI, CTL response index; CTL, cytotoxic T lymphocyte; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B envelope antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; I-NR, interferon nonresponders; I-PR, interferon partial responders; rIL-2, recombinant human IL-2; S-CR, spontaneous complete recovery; S-PR, spontaneous partial recovery.

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Table I. Characteristics of the Patients Studied

Patient	Sex/race	Age	HLA					
			A	A	B	B	C	C
Complete interferon-responders (HBsAg <sup>-</sup> , HBeAg <sup>-</sup> , normal ALT)								
I-CR-1	M/White	54	2	1	5	35	4	4
I-CR-2	F/White	48	2	30	13	50	6	6
I-CR-3	M/White	49	2	23	35	62	3	4
I-CR-4	M/White	30	2	2	8	38	7	7
I-CR-5	M/White	55	2	2	39	62	n.d.	n.d.
Partial interferon-responders (HBsAg <sup>+</sup> , HBeAg <sup>-</sup> , normal ALT)								
I-PR-6	M/White	39	2	3	35	56	1	4
I-PR-7	M/White	37	2	1	8	60	3	3
I-PR-8	M/White	34	2	24	7	35	4	7
I-PR-9	F/White	47	2	1	35	62	3	7
I-PR-10	M/White	67	2	3	7	62	3	7
Interferon-nonresponders (HBsAg <sup>+</sup> , HBeAg <sup>+</sup> or elevated ALT)								
I-NR-11	M/White	51	2	24	7	35	n.d.	n.d.
I-NR-12	M/Asian	33	2	24	35	70	4	4
I-NR-13	M/Asian	55	2	24	54	60	1	7
I-NR-14	M/White	41	2	39	60	60	7	8
Untreated, chronic hepatitis B (HBsAg <sup>+</sup> , HBeAg <sup>+</sup> , elevated ALT)								
UT-1	M/White	34	2	2	78	13	7	7
UT-2	M/White	57	2	68	59	44	5	7
UT-3	M/White	71	2	1	8	44	n.d.	n.d.
UT-4	M/White	n.k.	2	2	44		3	6
UT-5	M/White	41	2	69	41	52	n.d.	n.d.
UT-6	M/Asian	42	2	2	13	13	3	3
UT-7	M/Asian	32	2	68	75	50	1	1
UT-8	M/Asian	43	2	w33	58	46	n.d.	n.d.
Untreated, spontaneously completely recovered (HBsAg <sup>-</sup> , HBeAg <sup>-</sup> , normal ALT)								
S-CR-1	M/White	56	2	2	44	44	w3	w3
S-CR-2	M/White	52	2	26	51	17	n.d.	n.d.
Untreated, spontaneously partially recovered (HBsAg <sup>+</sup> , HBeAg <sup>-</sup> , normal ALT)								
S-PR-3	F/Hispanic	36	2	24	60	60	6	6
S-PR-4	M/Asian	45	2	24	7	75	7	8
Acute or postacute hepatitis B								
A-1	M/White	41	2	24	51	53	1	1
A-2	M/Asian	37	2	68	44	54	57	57
Uninfected controls								
N-1	M/White	28	2	2	51	51	1	1
N-2	F/Hispanic	49	2	24	7	27	2	7
N-3	M/Hispanic	48	2	2	39	51	7	7
N-4	F/Asian	35	2	24	27	77	3	8
N-5	M/Asian	51	2	24	35	72	4	4
N-6	M/Asian	36	2	29	44	44	7	8
N-7	F/Asian	33	2	3	35	49	4	7
N-8	M/Asian	53	2	2	68	68	3	3
N-9	F/Hispanic	31	2	2	18	35	4	5
N-10	F/White	38	2	32	18	60	3	7
N-11	F/White	40	2	11	35	61	3	4
N-12	F/White	39	2	29	27	52	1	7

n.d., not done; n.k., not known; ALT, serum alanine aminotransferase.

(spontaneous partial recovery: S-PR 3–4) or both HBeAg and HBsAg (spontaneous complete recovery: S-CR 1–2). All except one patient with a complete or partial response or spontaneous recovery had normal serum aminotransferases, and all except one of the untreated or nonresponder patients had raised values.

IFN-treated patients had received therapy as a part of controlled

trials of this agent (16–18) except I-NR-14 had been treated 5–11 yr before blood was sampled for this study (Table II). The two patients with acute hepatitis B were positive for HBsAg and HBeAg and had aminotransferases greater than 10 times the upper limit of the normal range during the acute illness (Table II). Blood for this study was sampled during the acute phase of infection in patient A-1 and during

Table II. Clinical, Biochemical, and Virological Evaluation at the Time of Immunological Analysis

Patient	Years after interferon	ALT (U/liter)*	HBV-DNA (bDNA) <sup>‡</sup> (PCR) <sup>§</sup>		HBsAg	HBeAg	anti-HBs	anti-HBe	anti-HBc	CTL response			
										wk 2		wk 3	
										(CRI)	(>18%)	No. peptides recognized	mean % cytotoxicity
<b>Complete interferon-responders (HbsAg<sup>-</sup>, HBeAg<sup>-</sup>, normal ALT)</b>													
I-CR-1	11	22	<0.7	(-)	(-)	(-)	(+)	(+)	(+)	614	(+)	3	65
I-CR-2	7	12	<0.7	(+)	(-)	(-)	(+)	(+)	(+)	234	(+)	2	40
I-CR-3	8	24	<0.7	(+)	(-)	(-)	(+)	(+)	(+)	328	(+)	2	35
I-CR-4	7	58	<0.7	(+)	(-)	(-)	(+)	(+)	(+)	0	(+)	1	38
I-CR-5	5	12	<0.7	(-)	(-)	(-)	(-)	(-)	(+)	0	(+)	1	38
<b>Partial interferon-responders (HBsAg<sup>+</sup>, HBeAg<sup>-</sup>, normal ALT)</b>													
I-PR-6	6	28	<0.7	(-)	(+)	(-)	(-)	(+)	(+)	0	(+)	2	85
I-PR-7	10	31	<0.7	(+)	(+)	(-)	(-)	(+)	(+)	397	(+)	2	54
I-PR-8	5	28	<0.7	(+)	(+)	(-)	(-)	(+)	(+)	0	(+)	2	78
I-PR-9	5	23	<0.7	(+)	(+)	(-)	(-)	(+)	(+)	0	(+)	2	51
I-PR-10	5	21	<0.7	(+)	(+)	(-)	(-)	(+)	(+)	0	(-)	0	0
<b>Interferon-nonresponders (HBsAg<sup>+</sup>, HBeAg<sup>+</sup> or elevated ALT)</b>													
I-NR-11	5	26	12.41	(+)	(+)	(+)	(-)	(-)	(+)	0	(-)	0	0
I-NR-12	7	56	<0.7	(+)	(+)	(-)	(-)	(+)	(+)	0	(+)	1	30
I-NR-13	1	147	<0.7	(+)	(+)	(-)	(-)	(+)	(+)	0	(-)	0	0
I-NR-14	1	50	622	(+)	(+)	(+)	(-)	(-)	(+)	0	(-)	0	0
<b>Untreated, chronic hepatitis (HBsAg<sup>+</sup>, HBeAg<sup>+</sup>, elevated ALT)</b>													
UT-1	—	85	1788	n.d.	(+)	(+)	(-)	(-)	(+)	0	(+)	1	19
UT-2	—	20	12348	n.d.	(+)	(+)	(-)	(-)	(+)	0	(+)	2	33
UT-3	—	123	281	n.d.	(+)	(+)	(-)	(-)	(+)	0	(+)	2	27
UT-4	—	421	n.a.	(+)	(+)	(+)	(-)	n.d.	(+)	0	(+)	2	46
UT-5	—	246	1438	n.d.	(+)	(+)	(-)	(-)	(+)	0	(-)	0	0
UT-6	—	247	981	n.d.	(+)	(+)	(-)	(-)	(+)	0	(-)	0	0
UT-7	—	86	65	n.d.	(+)	(+)	(-)	n.d.	(+)	0	n.d.	n.a.	n.a.
UT-8	—	85	20	n.d.	(+)	(+)	(-)	(+/-)	(+)	0	(-)	0	0
<b>Untreated, spontaneously completely recovered (HBsAg<sup>-</sup>, HBeAg<sup>-</sup>, normal ALT)</b>													
S-CR-1	—	13	<0.7	(-)	(-)	(-)	(+)	(+)	(+)	190	(+)	3	48
S-CR-2	—	18	<0.7	(+)	(-)	(-)	(+)	(-)	(+)	0	n.d.	n.a.	n.a.
<b>Untreated, spontaneously partially recovered (HBsAg<sup>+</sup>, HBeAg<sup>-</sup>, normal ALT)</b>													
S-PR-3	—	38	<0.7	(+)	(+)	(-)	(-)	(+)	(+)	152	(+)	3	26
S-PR-4	—	36	<0.7	(+)	(+)	(-)	(-)	(+)	(+)	291	(+)	0	0
<b>Acute or postacute Hepatitis B</b>													
A-1	—	844	0.757	(+)	(+)	(+)	(-)	(-)	(+)	1374**	n.d.	n.a.	n.a.
A-2	—	19	n.d.	(-)	(-)	(-)	(+)	n.a.	(+)	764	n.d.	n.a.	n.a.
<b>Uninfected controls</b>													
N-1	—	n.d.	n.d.	(-)	(-)	n.d.	(-)	n.d.	(-)	0	n.d.	n.a.	n.a.
N-2	—	n.d.	n.d.	(-)	(-)	n.d.	(-)	n.d.	(-)	0	n.d.	n.a.	n.a.
N-3	—	n.d.	n.d.	(-)	(-)	n.d.	(-)	n.d.	(-)	0	n.d.	n.a.	n.a.
N-4	—	n.d.	n.d.	(-)	(-)	n.d.	(-)	n.d.	(-)	0	n.d.	n.a.	n.a.
N-5	—	n.d.	n.d.	(-)	(-)	n.d.	(-)	n.d.	(-)	0	n.d.	n.a.	n.a.
N-6	—	n.d.	n.d.	(-)	(-)	n.d.	(-)	n.d.	(-)	0	n.d.	n.a.	n.a.
N-7	—	n.d.	n.d.	(-)	(-)	n.d.	(-)	n.d.	(-)	0	n.d.	n.a.	n.a.
N-8	—	n.d.	n.d.	(-)	(-)	n.d.	(-)	n.d.	(-)	0	(-)	0	0
N-9	—	n.d.	n.d.	(-)	(-)	n.d.	(-)	n.d.	(-)	0	(-)	0	0
N-10	—	n.d.	n.d.	(-)	(-)	n.d.	(-)	n.d.	(-)	0	(-)	0	0
N-11	—	n.d.	n.d.	(-)	(-)	n.d.	(-)	n.d.	(-)	0	(-)	0	0
N-12	—	n.d.	n.d.	(-)	(-)	n.d.	(-)	n.d.	(-)	0	(+)	1	35

\*ALT, serum alanine aminotransferase, normal range 0–41 U/liter for interferon-treated patients, 0–45 U/liter for all other patients; <sup>‡</sup> ×1,000,000 genome equiv./ml; <sup>§</sup> CRI derived from 4/5 peptides; n.d., not done; n.a., not applicable.

the convalescent phase, i.e., 16 mo after acute hepatitis in patient A-2. At the time of this study, serum alanine aminotransferase was elevated in patient A-1 and within normal range in patient A-2. While patient A-1 displayed high HBV-DNA levels as determined by bDNA analysis, patient A-2 had completely cleared HBV-DNA and was nonreactive even by the more sensitive nested PCR assay (Table II).

The 12 healthy controls were negative for HBsAg, anti-HBs, and HBV-DNA. All subjects were repeatedly anti-hepatitis C virus and anti-HIV negative.

**Synthetic peptides and HBV antigens.** A panel of peptides representing five previously identified HLA-A2-restricted HBV CTL epitopes ([2, 5, 6] and F.V. Chisari, unpublished results) was used in this study (Table III). The peptides contained free amine NH<sub>2</sub> termini and free acid COOH termini and were synthesized by the Core Laboratory of the Scripps Research Institute, La Jolla, CA, using FMOC Peptide Chemistry on a Gilson Multipetide Synthesizer. Recombinant (r) hepatitis B core antigen (HBcAg) was obtained from bacterial extracts of *Escherichia coli* as previously described (19).

**Recombinant expression vectors.** Recombinant vaccinia virus constructs encoding the large envelope protein (VHBS4, *adw* subtype), the HBV polymerase protein (Vpol, *ayw* subtype) (6, 20), or the HBV core protein (C-Vac, *ayw* subtype) (21) were used to induce transient expression of endogenously processed HBV proteins in human EBV-B cell lines (B-LCL) as previously described (22). Wt-vaccinia virus (21) was used as a control.

**Stimulation of PBMC with synthetic peptides.** Between 100 and 250 ml of whole blood was drawn from all patients in bags containing 35 ml CPD solution (920 mg sodium citrate, 892 mg dextrose, 105 mg citric acid, 77.7 mg sodium phosphate monobasic) and processed within 24 h. Cryopreserved, instead of freshly isolated, PBMC was used for the analysis of patients UT-1 to UT-5 only.

PBMC from patients and normal donors were separated on Ficoll-Histopaque density gradients (Sigma Chemical Co., St. Louis, MO), washed three times in Ca<sup>2+</sup>- and Mg<sup>3+</sup>-free PBS (GIBCO Laboratories, Grand Island, NY), resuspended in RPMI 1640 (GIBCO Laboratories), supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (10 µg/ml), and 10% heat-inactivated human AB serum and plated in 96-well round bottom plates at 0.4 × 10<sup>6</sup> cells/100 µl per well. Synthetic peptides were added at 10 µg/ml to eight replicate cultures per peptide. Recombinant HBcAg was added at 1 µg/ml as a source of T cell help (2, 5, 6) in all patients studied. On days 3 and 10, 100 µl of RPMI containing 10% (vol/vol) human AB serum and 10 U/ml recombinant human IL-2 (rIL-2) (Hoffmann-La Roche, Inc., Nutley, NJ) were added to each well. On day 7 the cultures were transferred into 96-well flat-bottom plates and restimulated with peptide, rIL-2 and 10<sup>5</sup> irradiated (3,000 rad) autologous PBMC feeder cells. Cultures were tested for cytotoxic activity in a split-well CTL-assay on day 14. The remaining cells in each set of eight replicate wells were combined, transferred into a single well of a 24-well plate, and stimulated with peptide, rIL-2, and 10<sup>6</sup> irradiated (3,000 rad) autologous feeder cells in a total vol of 1 ml. After addition of 1 ml complete medium with rIL-2 on day 18, these peptide-specific CTL lines were used on days 21–24 to further characterize the CTL response.

Table III. The Epitopes

Epitope number	Virus	Protein	Amino acid termini	Amino acid sequence	Reference
1	HBV	Core	18–27	FLPSDFPSPV	2
2	HBV	Env	183–191	FLLTRILTI	*
3	HBV	Env	335–343	WLSLLVPFV	5
4	HBV	Pol	455–463	GLSRVYVARL	6
5	HBV	Pol	575–583	FLLSLGIHL	6

\*F.V. Chisari, unpublished results.

**Cytotoxicity assay.** An HLA-A2.1, B7, Cw7-positive EBV-immortalized B cell line (JY), used for the production of target cells, was maintained in RPMI with 10% (vol/vol) heat-inactivated FCS (GIBCO Laboratories). Target cells were either incubated overnight with synthetic peptides at 10 µg/ml or infected with recombinant vaccinia viruses. Vaccinia-infected targets were prepared by infection of 10<sup>6</sup> JY-cells at an moi of 10 on a rocking plate at room temperature for 1 h followed by a single wash and overnight incubation at 37°C. Target cells were labeled with 100 µCi of <sup>51</sup>Cr (Amersham Corp., Arlington Heights, IL) for 1 h and washed four times with HBSS. Cytolytic activity was determined in a standard 4-h split-well <sup>51</sup>Cr release assay using U-bottomed 96-well plates with 3,000 targets/well. Stimulated PBMC from patients and normal controls were tested at E/T ratios of 30–50:1 on day 14 and at an E/T ratio of 60:1 on day 21–24 after induction of the culture. For each peptide eight replicate cultures were tested on day 14. Percent cytotoxicity was determined from the formula: 100 × [(experimental release – spontaneous release)/(maximum release – spontaneous release)]. Maximum release was determined by lysis of targets with 10% Triton X-100 (Sigma Chemical Co.). Spontaneous release was < 25% of maximum release in all experiments. In selected experiments, anti-CD4 and anti-CD8 antibodies purchased from Becton Dickinson Co. (San Jose, CA) were added to the effector cells, and anti-class I (W6/32) and anti-HLA-A2.1 antibody (MA 2.1) (American Type Tissue Collection, Rockville, MD) were added to the target cells at 10 µg/ml for 30 min before starting the assay.

**Limiting dilution analysis of CTL precursor frequency.** For quantitative analysis of epitope specific CTL precursors, varying numbers (0.5–4 × 10<sup>5</sup>) of PBMC plus 10<sup>5</sup> irradiated (3,000 rad) autologous PBMC were plated in individual wells of 96-well U-bottom plates in 100 µl RPMI 1640 (GIBCO Laboratories) containing 10 µg/ml peptide and 1 µg/ml recHBcAg and supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (10 µg/ml), and 10% heat-inactivated human AB serum. 24 replicate cultures were established at each responder cell concentration. Cultures were fed with 10 U/liter rIL-2 on days 4, 7, and 11 and restimulated with autologous, irradiated (3,000 rad) PBMC and 10 µg/ml peptide on day 7. A split-well CTL-assay was performed on day 14. Cultures were regarded as positive if the specific cytotoxicity was > 2 SD above the mean of the nonspecific cytotoxicity of all cultures and CTL precursor frequency was calculated using the maximum likelihood method as previously described (23).

**HBV-DNA determination.** HBV-DNA was quantitated using the bDNA nucleic acid hybridization assay Quantiplex™ HBV-DNA Assay (Chiron Corp., Emeryville, CA) according to the manufacturer's instructions. Results were expressed in 10<sup>6</sup> genome equivalents per ml.

For PCR analysis, HBV-DNA was extracted from serum and PBMC as previously described (24). Briefly, 100 µl of serum were incubated at 42°C for 3 h with proteinase K (50 µg/reaction) in 200 µl of lysis buffer containing 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA, pH 8.0, and 0.5% SDS. DNA was extracted with phenol-chloroform, precipitated with sodium acetate-ethanol, washed with 70% ethanol, and dissolved in 20 µl of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0). 5 µl of the DNA sample was used for PCR-amplification.

PBMC, cryopreserved in 80% FCS, 10% DMSO, 10% RPMI, and stored in liquid nitrogen until use, was thawed quickly, washed once in 15 ml HBSS, and incubated in 5 ml lysis buffer containing 50 µg proteinase K at 37°C overnight. The next day, DNA was extracted with phenol-chloroform, precipitated with sodium acetate-ethanol, washed with 70% ethanol, and dissolved in TE buffer. 0.2 µg of the DNA sample was used for PCR-amplification.

**HBV DNA detection using a nested PCR.** DNA isolated from serum and PBMC was analyzed by PCR using highly conserved diagnostic primer pairs that yielded a PCR product of 441 and 230 bp, respectively (HBV246S GAGTCTAGACTCGTGGTGGGA and HBV687AS CGA-ACCACTGAACAAATGGC as external primers and HBV370S

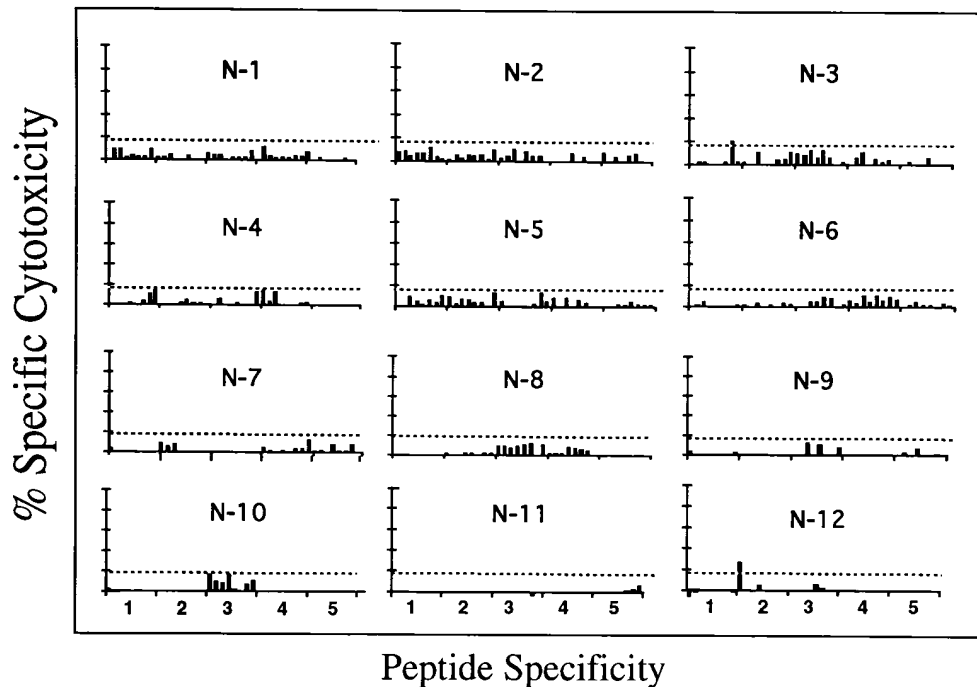


Figure 1. HBV-specific CTL response in healthy, uninfected control persons (N-1-12). PBMC were stimulated with 10  $\mu$ g/ml of peptide for 2 wk as described in Methods and tested in a 4-h  $^{51}$ Cr release assay against JY target cells prepulsed overnight with the same peptide. Results shown represent percentage of specific lysis in a 4-h  $^{51}$ Cr release assay at an E/T ratio of 40-50:1. The peptide specificity is shown in Table III. The dotted line indicates the percent specific cytotoxicity cut-off that was required in at least two of the eight replicate cultures per peptide to reach the definition of a positive CTL response.

TATCGCTGGATGTGTCTGC and HBV660AS CTGAGCCAG-GAGAAACGG as internal primers) 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, followed by 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  $\mu$ l of the mixture resulting from the direct reaction was amplified with the internal primer pair under the same conditions.

To eliminate sources of DNA contamination, DNA extraction and PCR were performed in separate rooms, pipetting devices were kept separately, and plugged pipette tips were used. All sera and PBMC samples were divided into small aliquots, 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 and/or PBMC of healthy, uninfected individuals and HBV-positive patients. Con-

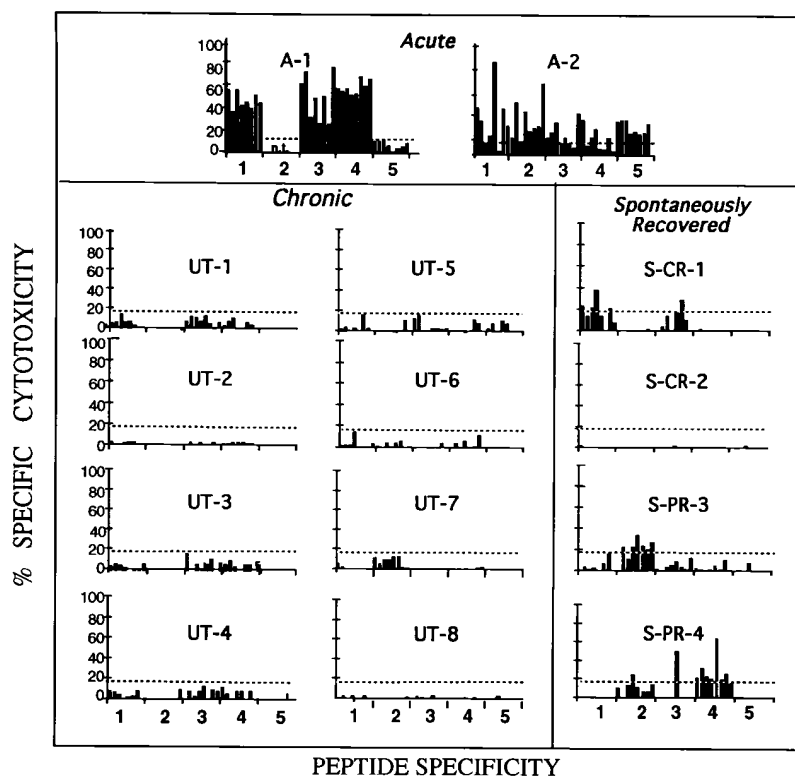


Figure 2. HBV-specific CTL response in patients with acute HBV one of whom (patients A-1) was currently infected and the other (patient A-2) had resolved the infection 16 mo earlier; eight untreated patients with chronic hepatitis (patients UT-1 to UT-8); and four patients who had completely (patients S-CR-1 and S-CR-2) or partially (patients S-PR-1 and S-PR-2) recovered from chronic hepatitis without antiviral or IFN therapy. PBMC were stimulated with 10  $\mu$ g/ml of peptide 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. Results shown represent percentage of specific lysis in a 4-h  $^{51}$ Cr release assay at an E/T ratio of 40-50:1. The peptide specificity is shown in Table III. The dotted line indicates the percent specific cytotoxicity cut-off required in at least two of the eight replicate cultures to define the CTL response to a given peptide as positive.

tamination controls consisted of water added to the PCR mixture instead of DNA.

## Results

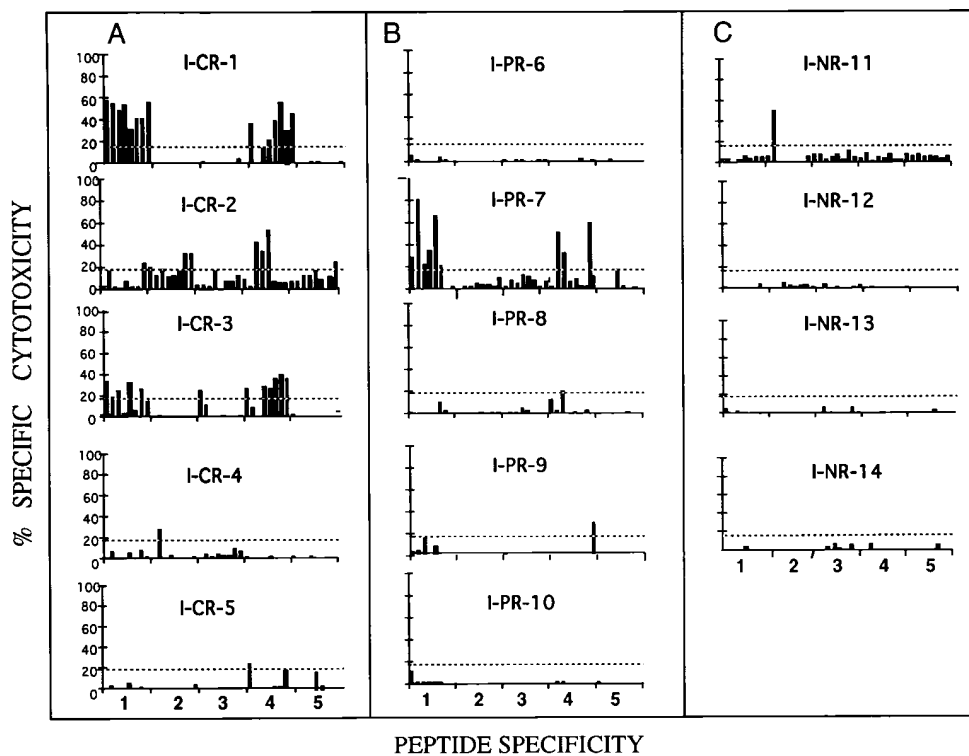
*An HBV-specific CTL response is detectable after resolution of chronic hepatitis.* Using a macrowell peptide stimulation technique to expand low frequency HBV-specific CTL precursors in the PBMCs of infected patients, we have previously shown that acutely infected patients produce an easily detectable peripheral CTL response to HBV and that chronically infected patients do not (3–6). In the current study we used a miniaturized modification of this technique (Rehermann, B., C. Ferrari, and F. V. Chisari, manuscript submitted for publication) to permit the analysis of multiple replicate assays for each peptide, thereby establishing a database needed to define a “normal range” of irrelevant cytotoxicity. Based on inspection of the responses observed in 12 uninfected normal control subjects using this stimulation technique (Fig. 1), we required at least two of the eight replicate cultures stimulated by a given peptide to display specific cytotoxicity of > 18% for the CTL response to that peptide to be scored as positive. This level of cytotoxicity was 6 SD ( $4 \times 3.8\%$ ) above the mean (2.3%) percent cytotoxicity detected for all of the peptide-stimulated cultures in the normal control group (Fig. 1, *dashed line*).

Using these criteria among two patients with acute hepatitis B (tested during the acute or convalescent phase of disease) vigorous CTL responses were detected in both (Fig. 2). Using the working criteria for a positive CTL response, specific cytotoxicity was present against three of four peptides in patient A-1 and five of five peptides in patient A-2. Furthermore 57 of the 72 individual cultures yielded CTL responses above the cut off of 18%, and in many instances the cytotoxicity was quite high (up to 85%). The differential responsiveness of the pa-

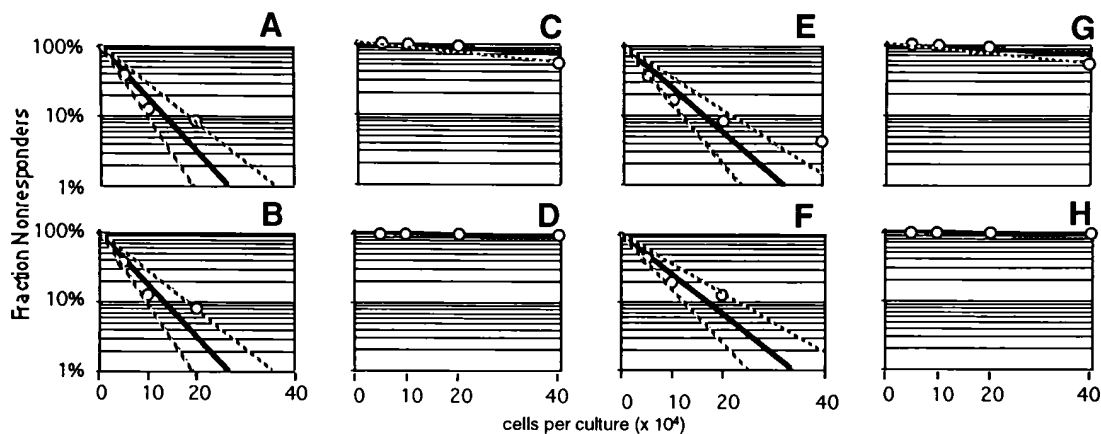
tients and controls was not due to the use of HBcAg as a source of T cell help, since we have previously reported the same selectivity of CTL responsiveness in patients when tetanus toxoid was substituted for HBcAg (25).

In contrast to patients with acute hepatitis, none of the eight patients with chronic hepatitis B who had not been treated (UT 1–8) achieved the criteria for a positive CTL response, and among 320 individual assays, none were above the cut off of 18% with this panel of epitopes (Fig. 2). These results using this microcytotoxicity assay system are in accordance with previous reports demonstrating that patients with acute hepatitis B have a multispecific CTL response against HBV whereas patients with chronic hepatitis B have CTL responses that are minimally above background or that of a normal population (3, 5, 6, 26).

In contrast to the chronically infected patients, however, three of the four patients who had previously spontaneously cleared HBeAg (S-CR-1, S-PR-3, and S-PR-4) displayed a CTL response to at least one of the HBV epitopes studied (Fig. 2), and 20 out of 160 CTL assays were above the cut off of 18%. Similarly, three of the five patients who completely responded to IFN therapy with normalization of serum alanine aminotransferase activity and loss of HBeAg and HBsAg (patients I-CR-1, I-CR-2, and I-CR-3) and one of the five partial responders (I-PR-7) also displayed a peripheral blood CTL response against epitopes in the HBV core, envelope, and polymerase proteins (Fig. 3). A total of 47 out of 400 CTL assays was above the cut-off in the group of patients responding to IFN. In contrast, none of the IFN nonresponders displayed a significant response CTL against these HBV epitopes (Fig. 3), and only 1 out of 160 CTL assays was above the cut-off. It is noteworthy that the CTL response was detectable 8–11 yr after IFN therapy in the treated patients even though they had cleared HBV DNA as detected by the branched chain DNA



*Figure 3.* HBV-specific CTL response after 2 wk of in vitro peptide stimulation in chronic HBV patients who responded either completely (patients I-CR-1 to I-CR-5) or partially (patient I-PR-6 to I-PR-10) to IFN therapy. Patients I-NR-11 to I-NR-14 did not respond to IFN. PBMC were stimulated with 10  $\mu\text{g}/\text{ml}$  of peptide for 2 wk as described in Methods and tested in a 4-h  $^{51}\text{Cr}$  release assay against JY target cells prepulsed overnight with the same peptide. Results shown represent percentage of specific lysis in a 4-h  $^{51}\text{Cr}$  release assay at an E/T ratio of 40–50:1. The peptide specificity is shown in Table III. The dotted line indicates the percent specific cytotoxicity cut-off (18%) required in at least two of the eight replicate cultures to define the CTL response to a given peptide as positive.



Panel	Patient	Category	Epitope	CTL Precursors	95% Confidence Interval
A	A-1	Acute Hepatitis B	Pol455-463	1/55,000	1/40,000-1/75,000
B	A-1	Acute Hepatitis B	Env335-343	1/69,000	1/52,000-1/93,000
C	UT-1	untreated, chronic hepatitis B	Pol455-463	1/708,000	1/460,000-1/1,089,000
D	UT-1	untreated, chronic hepatitis B	Env335-343	<1/5,830,000	*
E	I-CR-3	Complete IFN-responder	Pol455-463	1/70,000	1/52,000-1/95,000
F	I-CR-3	Complete IFN-responder	Env335-343	1/74,000	1/54,000-1/102,000
G	I-PR-8	Partial IFN-responder	Pol455-463	<1/909,890	*
H	I-NR-12	IFN-Nonresponder	Env335-343	<1/5,826,429	*

Figure 4. Analysis of the CTL precursor frequency of selected responses. Peptide specific CTL precursor frequencies were determined after 2 wk of peptide stimulation by limiting dilution analysis as described in Methods. The results are shown as semilogarithmic plot of responder cell input per well against the percentage of negative wells, and also in tabular form. CTL precursors are expressed as number of peptide-specific CTL precursors in  $10^6$  PBMC. \*CTL precursor frequency too low to establish a confidence interval.

assay. Indeed traces of HBV DNA were detectable in either serum or PBMC using the nested PCR technique in most of these individuals (Table II).

*The strength of the CTL response is similar after resolution of acute and chronic hepatitis.* The strength of the CTL response was estimated semiquantitatively by calculating the CTL response index (CRI) for each patient. The CRI is an arbitrary scoring system that represents the sum of all the specific cytotoxicity percentages greater than the cut off value of 18% for peptides that scored positive after 2 wk of in vitro stimulation. The CRI provides an estimate of the relative strength of the overall CTL response to the panel of peptides in a given patient. In selected cases, the strength of the CTL response was also quantitated by measuring the CTL precursor frequency displayed by the patient for specific viral peptides. As shown in Table II, the highest CRIs were detected in the two acutely infected patients (CRIs = 1,374 and 764). The CRI results were intermediate in the four patients with a complete response to IFN (CRIs = 234–614) and were lower among those who had spontaneously cleared HBeAg (CRIs = 152–291).

Thus, it would appear that the CTL response is nearly as strong after clearance of chronic HBV infection as it is after clearance of acute infection. To confirm this impression, the number of HBV-specific CTL precursors in the peripheral blood of representative patients were quantitated by limiting dilution analysis (LDA). As can be seen in Fig. 4 E and F, pa-

tient I-CR-3 displayed CTL precursor frequencies of 1/55,000 and 1/67,000 PBMC for epitopes Env 335-343 and Pol 455-463, respectively. This is comparable to the CTL precursor frequency for the same peptides after resolution of acute hepatitis in patient A-2 (Fig. 4 A and B), and it is 1–2 orders of magnitude higher than in untreated patient UT-16 with chronic hepatitis B (Fig. 4 C and D) and in the IFN nonresponder I-NR-12 (Fig. 4 H).

*Prolonged peptide stimulation unmasks CTL responses in chronically infected patients.* The foregoing results (Figs. 1–4) were obtained with PBMC that had been stimulated in vitro with peptides for 2 wk. To enhance the sensitivity of the system, the lymphocytes remaining in the microwells after cytotoxicity analysis were pooled, restimulated for a third week with the corresponding peptide, and tested for peptide-specific CTL activity. Using this protocol, most of the CTL responses that could be retested were confirmed and new CTL responses were detected (Figs. 5 and 6 and Table II). Most importantly, CTL responses were detected in three of the untreated patients with ongoing chronic hepatitis that were tested in this manner (Fig. 5) and in almost all of the partial IFN-responders (Fig. 6).

As summarized in Table IV, after 3 wk of in vitro stimulation 15/24 possible CTL responses (63%) were detected in the group of patients who completely recovered either spontaneously or after IFN therapy, and 12/35 possible CTL responses

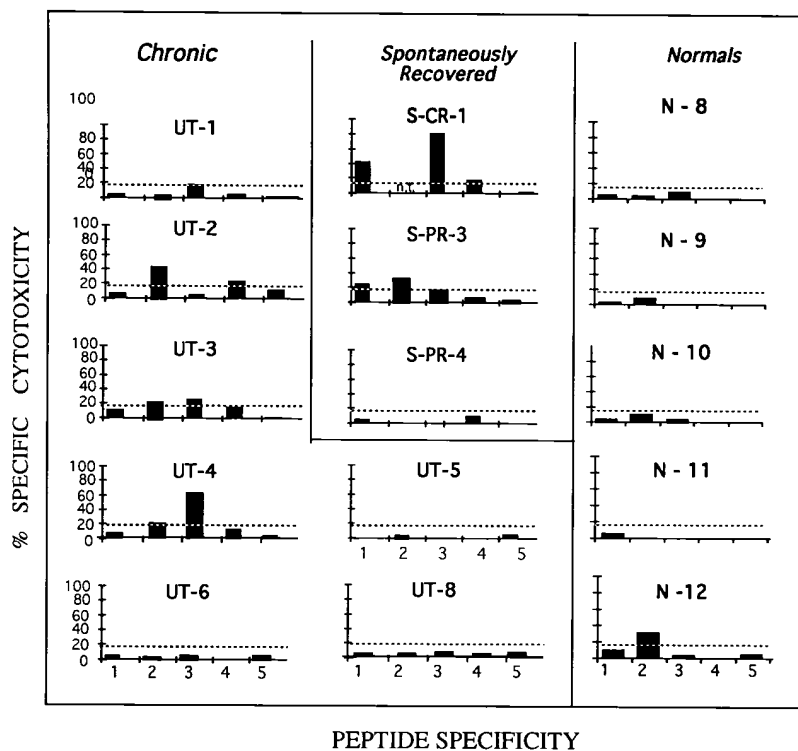


Figure 5. HBV-specific CTL response after 3 wk of in vitro peptide stimulation. PBMC were stimulated with 10  $\mu$ g/ml of peptide for 2 wk in eight replicate cultures per peptide, pooled, restimulated on day 14, and tested in a 4-h  $^{51}$ Cr release assay against JY target cells prepulsed overnight with the same peptide as described in Methods. Results shown represent percentage of specific lysis in a 4-h  $^{51}$ Cr release assay at an E/T ratio of 60:1. The peptide specificity and the dashed line are as described in the legend for Fig. 1 (N-8 to N-12, normal, uninfected controls); the patient groups are as described in the legend to Fig. 2.

(34%) were observed in the group of patients who recovered partially. Most importantly, 8/40 of the possible responses (20%) developed in the untreated patient group. In contrast, only 7 and 4% (one response per group) of the possible responses were detected in IFN-nonresponders and in the healthy, uninfected control group, respectively, when tested after 3 wk of in vitro stimulation. As shown in Table IV, the same hierarchy was observed when the mean percent cytotoxicity for the peptides that scored as positive was compared in these groups; i.e., it was highest in the complete responders ( $47 \pm 19\%$ ) followed by the partial responders ( $34 \pm 27\%$ ) and lowest in the untreated patients ( $18 \pm 16\%$ ).

This late-developing CTL activity probably reflects a low initial precursor frequency that requires a longer expansion time in vitro to reach detectable levels. The detectability of a CTL response in the untreated patients with chronic hepatitis is compatible with its enhancement after IFN therapy, and it suggests that HBV-specific CTL are present in the peripheral blood during chronic HBV infection but that their frequency is too low to terminate the infection.

*The cytotoxic T cells recognize endogenously processed viral antigens.* Peptide-stimulated CTL lines were tested for the ability to recognize endogenously synthesized antigen. Fig. 7 shows representative results of varying the E/T ratio and the type of target cells in CTL assay in two patients with chronic hepatitis B who had a long term complete response. A CTL line from patient I-CR-1 which was specific for Pol 455-463 also recognized endogenously synthesized HBV polymerase antigen. Similarly, a CTL line from patient I-CR-2 specific for the HBVenv 183-191 peptide recognized endogenously synthesized HBsAg. Both peptide-pulsed and vaccinia-infected target cells were lysed efficiently at E/T ratios as low as 3.7:1 for patient I-CR-1 and as low as 30:1 for patient I-CR-2 (Fig. 7, A and B). Control target cells without peptide were not signifi-

cantly lysed at these E/T ratios, nor were cells infected by a wild-type vaccinia virus used as control.

*The CTL response is Class I and HLA-A2 restricted and mediated by CD8 positive T cells.* To further characterize the cell population responsible for the CTL response observed in chronically infected patients that resolved the disease, we blocked the peptide recognition of specific cell lines with a panel of antibodies. As shown in Fig. 7, C and D, antigen recognition by the HBcAg 18-27 and HBsAg 335-343 specific CTL lines from patient I-CR-1 and the HBsAg 335-343 specific CTL line from patient I-CR-5 could be efficiently blocked by antibodies targeted against class I or HLA-A2 molecules. Antigen recognition was also significantly inhibited when antibodies against CD8, but not CD4, were incubated with the effector cells before the addition of target cells. These results confirm that the observed CTL response is HLA-A2 restricted and mediated by CD8 positive T cells.

## Discussion

It has been amply demonstrated that patients with acute hepatitis B virus infection produce a strong, polyclonal, and multi-specific CTL response to HBV while patients with chronic hepatitis do not (3, 5, 6). Despite recovery from acute hepatitis, clearance of viral antigens, and appearance of neutralizing antibodies, however, trace amounts of HBV DNA and virus-specific CTL often persist in the blood for decades (24) (Rehermann, B., C. Ferrari, and F. V. Chisari, manuscript submitted for publication) presumably reflecting persistence of a small viral reservoir that actively maintains the CTL response and is controlled by it. In chronically infected patients, however, this balance obviously favors the virus since the peripheral blood CTL response is very weak or not detectable, and the virus persists at titers many orders of magnitude higher than that ob-



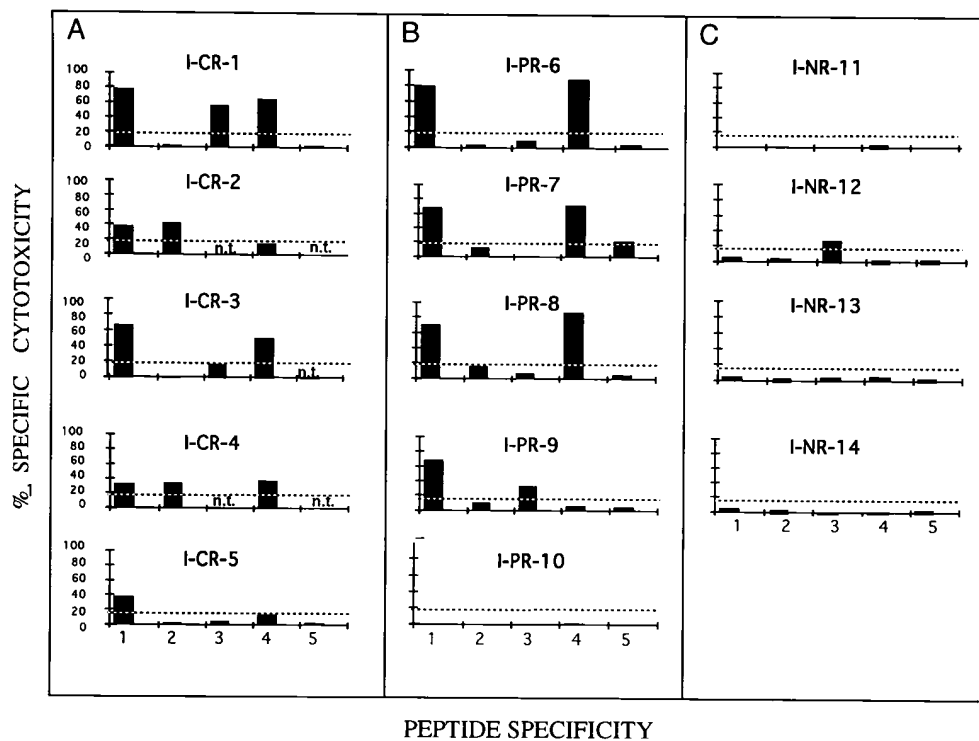


Figure 6. HBV-specific CTL response after 3 wk of in vitro peptide stimulation. The patient and peptide specificity is as described in the legend for Fig. 2.

served after recovery from acute infection (24, 27, 28). The mechanisms responsible for this imbalance between CTL response and virus that may determine the outcome of infection are not well understood. Elucidation of these mechanisms would contribute to an understanding of the immunological basis for viral persistence during chronic HBV infection and to the development of therapeutic strategies to enhance the HBV-specific immune response in chronically infected patients in the hope of terminating the infection.

The current study demonstrates that CTL specific for at least five epitopes in the HBV nucleocapsid, envelope and polymerase proteins are present in the peripheral blood of patients with chronic hepatitis but that their frequency is low, suggesting that they are quantitatively insufficient to mediate

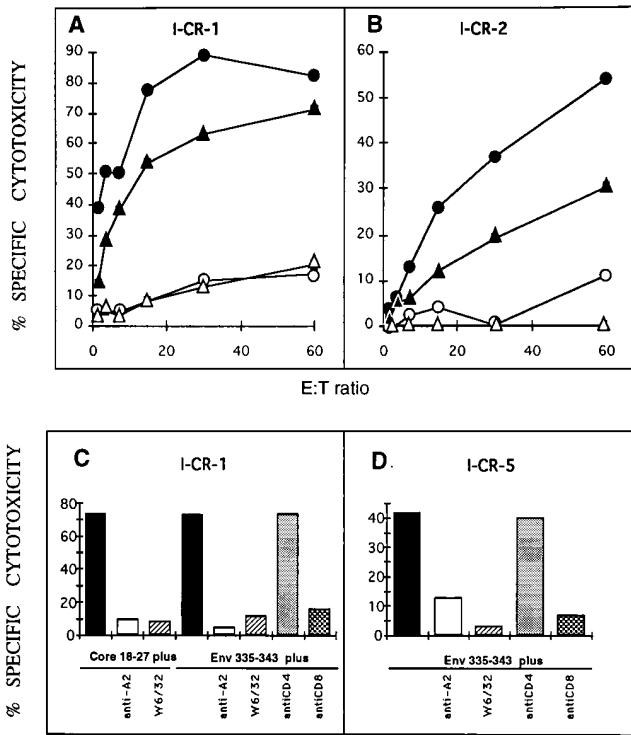
viral clearance but probably contribute to the liver disease in these individuals. Most importantly, the data indicate that these CTL can be activated and expanded in vivo in those chronically infected patients who experience spontaneous or IFN-induced viral clearance and disease remission. Furthermore, the CTL response appears to be as strong in these individuals as it is after resolution of acute hepatitis, and to display the same epitope specificity, functional characteristics, and inability to completely eradicate all traces of viral DNA from the blood. Thus, there is reason to believe that specific therapeutic stimulation of the CTL response to HBV may lead to viral clearance and disease remission in patients with chronic hepatitis. The basis for CTL nonresponse in the remaining patients is not clear and deserves further investigation while it is possible.

However, inspection of Table II illustrates several important relationships between the CTL response and clinical, serological, or virological parameters in these patients that could weaken the pathogenetic significance of these observations. For example, all of the patients who displayed a CTL response to HBV after 2 wk of in vitro stimulation were anti-HBe positive, and most of them were also anti-HBs positive (Table II). While these associations strengthen confidence in the validity of the CTL analysis in these patients, they raise the possibility that the reduction in viral titer in these patients could be due to the combined effects of the CTL and the antibodies, especially the anti-HBs, not just the CTL alone.

Table II also reveals that HBV-specific CTL was detectable (after 2 wk of in vitro stimulation) in < 100% of the patients who experienced spontaneous or IFN-induced remissions of chronic hepatitis. This raises the possibility that the CTL response may have been secondary to a reduction in viral load rather than causing it. This would be especially germane in the IFN-treated patients where CTL-independent antiviral events could have been initiated by the therapy. Since we did not have the opportunity to examine those patients before and

Table IV. Summary of the Cytotoxic T Cell Response after 3 wk of the Cell Culture

Patient group	CTL responses	Specific cytotoxicity	
		Mean	SD
Complete responders (I-CR and S-CR)	15/24 63%	47%	19%
Partial responders (I-PR and S-PR)	12/35 34%	34%	27%
Untreated patients (UT)	8/40 20%	18%	16%
Nonresponders (I-NR)	1/15 7%	30%	—
Normal controls (N)	1/25 4%	35%	—



**Figure 7.** (A and B) Recognition of endogenously synthesized antigen. Epitope-specific lines were generated by stimulating PBMC from patients I-CR-1 and I-CR-2 with 10  $\mu$ g/ml of peptide Pol455-463 and 10  $\mu$ g/ml of peptide Env183-191, respectively as described in Methods. Peptide-specific lines were tested for specific cytotoxicity against JY-target cells either pulsed with 10  $\mu$ g/ml peptide (●) (Pol455-463 for A, Env183-191 for B) or infected with recombinant vaccinia virus vPol (A) or vHBs4 (B), respectively, (▲) in a standard 4-h  $^{51}$ Cr release assay at varying E/T ratios. Wild type-vaccinia virus (△) or JY targets without peptide (○) were used as control. (C and D) Anti-HLA-A2 (MA 2.1) or anti-class I (W6/32) mAbs (American Type Tissue Collection) were added to peptide-pulsed JY target cells and anti-CD4 or anti-CD8 mAbs were added to the corresponding epitope-specific CTL lines at 10  $\mu$ g/ml 30 min before mixing effectors and target cells as described in Methods. Cytotoxicity was measured at an E/T ratio of 60:1 in a 4-h  $^{51}$ Cr release assay.

during the course of IFN therapy, we cannot be sure that the CTL response became detectable before the decrease in viral burden and clearance of viral antigens, or whether it appeared afterwards. However, we favor the first option because of a previous report that activation of HBV-specific CD4-positive helper T cells precedes the resolution of chronic hepatitis (13, 14).

Despite these caveats, we believe the current results suggest that the CTL probably contributed to the spontaneous or IFN-induced remissions in these patients for several reasons. First, the strength of the CTL response in these patients was comparable to that observed in patients who had recovered from acute HBV infection in this (Fig. 1) and previous studies (Rehermann, B., C. Ferrari, and F.V. Chisari, manuscript submitted for publication). Second, CTL activity was detectable in virtually all of the patients after an extra week of in vitro stimulation. Third, the strength of these late-onset CTL responses in the patients who experienced remissions was generally stronger than the late-onset CTL responses observed in the untreated patients and the single IFN nonresponder who dis-

played a CTL response to a single epitope after 3 wk of in vitro stimulation (Table II).

The results of this study have important implications for the development of immunotherapeutic approaches to terminate HBV infection in chronically infected patients. The idea that therapeutic enhancement of the HBV-specific CTL response could lead to viral clearance and disease remission in chronic hepatitis is not a new concept (29). Until now, however, this hypothesis has been based on inference from indirect evidence. The current results demonstrating that a strong CTL response commensurate with viral clearance is detectable in patients after resolution of chronic hepatitis establish a factual basis for the foregoing hypothesis. In addition, they raise a cautionary note suggesting that clearance of HBV and remission of liver disease may require more than enhancement of the CTL response and that the induction of neutralizing anti-HBs antibodies may also be needed for viral clearance to occur.

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