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Differential Cytotoxic T-Lymphocyte Responsiveness to the Hepatitis B and C Viruses in Chronically Infected Patients†

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Cytotoxic T lymphocytes (CTL) are thought to control hepatitis B virus (HBV) infection, since they are readily detectable in patients who clear the virus whereas they are hard to detect during chronic HBV infection. In chronic hepatitis C virus (HCV) infection, however, the virus persists in the face of a CTL response. Indeed, most infected patients respond to one or more HCV-1 (genotype 1a)-derived CTL epitopes in the core, NS3, and NS4 proteins, and the CTL response is equally strong in patients infected by different HCV genotypes, suggesting broad cross-reactivity. To examine the effect of the HCV-specific CTL response in patients with chronic hepatitis C on viral load and disease activity, we quantitated the strength of the multispecific CTL response against 10 independent epitopes within the HCV polyprotein. We could not detect a linear correlation between the CTL response and viral load or disease activity in these patients. However, the CTL response was stronger in the subgroup of patients whose HCV RNA was below the detection threshold of the HCV branched-chain DNA assay than in branched-chain-DNA-positive patients. These results suggest that the HCV-specific CTL response may be able to control viral load to some extent in chronically infected patients, and they indicate that prospective studies in acutely infected patients who successfully clear HCV should be performed to more precisely define the relationship between CTL responsiveness, viral clearance, and disease severity in this infection.

The hepatitis B and C viruses (HBV and HCV) are parenterally transmitted hepatotropic viruses that cause acute and chronic liver disease that may progress to hepatocellular carcinoma (10). Since neither virus is directly cytopathic, the class I-restricted cytotoxic T-lymphocyte (CTL) response is assumed to play a pivotal role in viral clearance and disease pathogenesis during HBV and HCV infections.

We have previously demonstrated that patients with acute HBV infection characteristically develop a vigorous, polyclonal, and multispecific HLA class I-restricted peripheral blood CTL response to several epitopes in the HBV envelope, nucleocapsid, and polymerase proteins (5, 17, 20). However, in chronically infected patients the peripheral CTL response to HBV is usually very difficult to detect. On the basis of these observations, we have suggested that the HLA class I-restricted CTL response to these viral epitopes plays a critical role in viral clearance during acute HBV infection and that the failure to mount a strong response could be the principal cause of HBV persistence.

This paradigm is not repeated in HCV infection, since chronically infected patients mount a polyclonal and multispecific CTL response to several HCV-encoded proteins (4, 8, 15, 16, 22). In the current study, we show that patients with a strong multispecific CTL response to HCV are more likely to have lower viral load and more likely to have a low level of serum alanine aminotransferase (ALT) activity. We also show that the CTL response is not enhanced by interferon therapy, which does reduce viral load and liver disease activity, apparently by CTL-independent mechanisms. The data imply that while HCV is not completely eradicated by the immune response in chronically infected patients, it is responsive to CTL-mediated control, suggesting that antigen-specific enhancement of the CTL response may have therapeutic benefit in chronic HCV infection.

MATERIALS AND METHODS

Patient populations. Totals of 36 HLA-A2-positive patients with chronic hepatitis C (Table 1), 8 HLA-A2-positive patients with chronic hepatitis B (Table 2), 2 HLA-A2-positive patients with acute hepatitis B (Table 2), and 23 HLA-A2-positive healthy, uninfected subjects (Table 3) were studied. All subjects were anti-human immunodeficiency virus (HIV) negative.

Diagnosis of HCV infection was based on standard clinical parameters and serological assays with the second-generation (c200/c22-3) Ortho HCV enzyme-linked immunosorbent assay test system (Ortho Diagnostics, Inc., Raritan, N.J.). Twenty-five HCV-infected patients (HCV-8 and HCV-13 to HCV-36) underwent diagnostic liver biopsy before interferon therapy, and all of them had histological evidence of chronic hepatitis. As shown in Table 1, 12 patients (HCV-1 to HCV-12) had not been treated with interferon or any other antiviral drugs. Ten patients (HCV-13 to HCV-22) were studied both before and also during or after interferon therapy. An additional 14 patients (HCV-23 to HCV-36) were studied only after therapy.

Patients who were treated with interferon received between 3 and 10 million U of either alpha interferon 1 (Interferon Sciences), alpha interferon n1 (Burroughs Wellcome), polyethylene glycol alpha interferon 2a (Hoffmann-La Roche, Inc.), alpha interferon 2b (Schering Corp.), or *r*-Met-interferon-consensus 1 (Amgen Corp.) subcutaneously on a weekly (polyethylene glycol alpha interferon 2a) or thrice weekly (all others) basis. In selected cases (patients HCV-15 to HCV-17, HCV-19, HCV-24, HCV-26, and HCV-33 to HCV-36), interferon therapy was preceded by a 6-week course of oral prednisone (60, 40, and 20 mg each for 2 weeks). Five patients (HCV-15, HCV-18, HCV-21, HCV-24, and HCV-26) responded to interferon therapy by normalization of ALT activity. While normalization of ALT was only temporary in patients HCV-15 and HCV-18, it was sustained for more than 6 months after cessation of interferon therapy and was accompanied by loss of viral RNA as determined by PCR

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TABLE 1. Characteristics of HCV-infected patients[a]

Patient	Age	Sex	HCV genotype	Value for samples before interferon therapy				Value for samples during or after interferon therapy			
				Total CRI	HCV RNA titer		ALT (U/liter)	Total CRI	HCV RNA titer		ALT (U/liter)
					bDNA	PCR			bDNA	PCR	
HCV-1	53	M	1b	1,043	95.7		44				
HCV-2	41	M	2b	535	<3.5	2	42				
HCV-3	40	M	1a	1,046	95.4		49				
HCV-4	46	F	2b	397	36.6		53				
HCV-5	43	M	ND	875	<3.5	Neg	69				
HCV-6	56	M	2a/2b	643	54.7		32				
HCV-7	26	F	ND	895	44.4		46				
HCV-8	38	M	1a/1b	708	260.2		102				
HCV-9	32	F	ND	892	34.7		62				
HCV-10	54	M	1b	440	54.3		31				
HCV-11	31	M	3a	1,054	<3.5	3	53				
HCV-12	41	M	ND	1,611	<3.5	Neg	52				
HCV-13	40	M	3a	693	50.3		419	577	ND	ND	171
HCV-14	47	M	2b	802	19.4		131	357	<3.5	2	61
HCV-15	37	M	1a	705	5.1		155	475	70.1		29
HCV-16	28	M	1b	417	120		81	254	41.7		102
HCV-17	41	M	1a	682	110		226	813	68		166
HCV-18	42	F	1b	526	113.5		357	224	ND		35
HCV-19	61	F	1a	333	60.8		190	99	63		182
HCV-20	36	M	1a	967	498.5		135	87	10.8		150
HCV-21	43	M	1a	897	21.9		126	1,002	<3.5	Neg	23
HCV-22	33	F	2b	1,001	44.4		74	794	<3.5	2	58
HCV-23	40	M	3a					675	<3.5	1	54
HCV-24	58	M	2b					501	<3.5	Neg	24
HCV-25	46	M	1b					328	22.7		58
HCV-26	42	F	1a					694	<3.5	Neg	29
HCV-27	39	M	1a					565	23.5		73
HCV-28	51	M	1a					1,000	77		159
HCV-29	53	M	2a					260	7.5		68
HCV-30	38	F	1b					359	66.9		74
HCV-31	51	M	2a					14	29.2		50
HCV-32	36	M	1b					1,769	7.3		159
HCV-33	34	M	2b					1,543	<3.5	1	ND
HCV-34	40	M	3a					1,324	<3.5	4	82
HCV-35	37	M	3a					555	29.9		49
HCV-36	31	M	1a/1b					1,071	<3.5	0	53

[a] Total CRI, sum of all of the specific cytotoxicity values for all epitopes in each patient. bDNA, bDNA assay for HCV RNA quantitation in 100,000 genome equivalents per ml. Assay sensitivity is 350,000 genome equivalents per ml of serum. Semiquantitative PCR with serial 10-fold serum dilutions of cDNA was performed when HCV RNA was not detected by bDNA analysis. The negative logarithm of the highest 10-fold dilution that was positive is indicated as HCV-RNA titer. For ALT, the upper limit of normal was 50 U/liter. Negative, PCR negative and undiluted; ND, not done; M, male; F, female.

for HCV-24 and HCV-26. Patient HCV-21 was lost to follow-up after interferon therapy and could not be assessed for long-term response.

Diagnosis of acute hepatitis B (Table 2) was based on clinical and biochemical evidence of self-limited acute liver injury according to standard diagnostic criteria, i.e., jaundice and serum ALT activity at least 15 times greater than the upper limit of normal, together with serological evidence of acute HBV infection, i.e., hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), and immunoglobulin M anti-HBc antibody, and the absence of serologic evidence of hepatitis delta virus or HCV infection (Abbott Laboratories, North Chicago, Ill.).

All patients with chronic hepatitis B (Table 2) were repeatedly serologically positive for HBsAg for more than 6 months. Four of these patients displayed mildly to moderately elevated serum ALT activity, and five patients displayed histological evidence for chronic active hepatitis. None of these patients had been treated with interferon at the time of this study.

Control subjects (Table 3) whose peripheral blood mononuclear cells (PBMC) were stimulated with HBV or HCV peptides had no clinical history of HBV or HCV infection, respectively, although 10 may have been occupationally exposed to blood products (i.e., as nurse, laboratory technician, fire fighter, etc.). Six of the controls whose PBMC were stimulated with HBV peptides (N-7, N-9, and N-19 to N-22) had been previously immunized with the HBV vaccine and displayed anti-HBsAg antibody titers. At the time of analysis, all controls were negative for HBsAg and for antibodies to HBcAg, HCV, and HIV. The study protocol was approved by the Human Subjects Committee at the Scripps Clinic and Research Foundation.

TABLE 2. Characteristics of HBV-infected patients

Patient[a]	Age	Sex[b]	ALT (U/liter)[c]	HBsAg	HBeAg	Anti-HBcAg	Anti-HBsAg	Anti-HBeAg	Liver biopsy result[d]
aHBV-1	38	M	844	+	+	+	−	−	
aHBV-2	34	M	3,822	+	−	+	−	−	
cHBV-1	49	M	49	+	+	+	−	−	CAH
cHBV-2	57	M	17	+	+	+	−	−	CAH
cHBV-3	NA[e]	M	27	+	−	+	−	ND[f]	ND
cHBV-4	32	M	102	+	+	+	−	ND	CAH
cHBV-5	38	M	34	+	−	+	−	−	CAH
cHBV-6	36	M	85	+	+	+	−	−	ND
cHBV-7	41	M	137	+	+	+	ND	+	CAH
cHBV-8	66	M	84	+	+	+	−	−	ND

[a] aHBV, acute HBV infection; cHBV, chronic HBV infection.
[b] M, male.
[c] Upper limit of normal, 50 U/liter.
[d] CAH, chronic active hepatitis.
[e] NA, not available.
[f] ND, not done.

TABLE 3. Uninfected controls

Control	Age	Sex[a]	Occupational risk
Controls used for HCV study[b]			
N-1	52	F	No
N-2	41	F	Yes
N-3	31	F	No
N-4	40	F	Yes
N-5	54	F	No
N-6	46	M	Yes
N-7	34	M	No
N-8	33	M	No
N-9	31	M	Yes
N-10	52	F	No
N-11	40	M	Yes
N-12	40	M	No
N-13	36	F	Yes
N-14	44	M	Yes
N-15	40	M	No
N-16	43	F	No
N-17	41	M	No
N-18	33	F	No
Controls used for HBV study[c]			
N-2	52	F	No
N-7	41	F	Yes
N-9	31	F	No
N-19	40	F	Yes
N-20	54	F	No
N-21	46	M	Yes
N-22	34	M	No
N-23	33	M	No

[a] F, female; M, male.
[b] All subjects were anti-HCV and anti-HIV negative.
[c] All subjects were HBsAg, anti-HBcAg, and anti-HIV negative.

Synthetic peptides and HBV antigens. Two panels of peptides representing 10 previously identified HLA-A2-restricted HCV CTL epitopes based on the HCV-1 sequence (genotype 1a) (4, 8, 22), and 5 HBV CTL epitopes (5, 17, 20, 21) were selected for this study (Table 4). All peptides were synthesized with a free-amine N terminus and free-acid C terminus by Chiron Mimotopes (Clayton, Australia). Lyophilized peptides were reconstituted at 20 mg/ml in dimethyl sulfoxide (Mallinckrodt, Paris, Ky.) and diluted to 1 mg/ml with RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.). Recombinant HBcAg (rH-BcAg) was obtained from bacterial extracts of *Escherichia coli* as previously described (18).

TABLE 4. The epitopes[a]

Epitope designation	Virus	Protein	Amino acid termini	Amino acid sequence	Reference(s)	Mean control CRI-P value (+3 SD)
1	HCV	Core	35–44	YLLPRRGPRL	4, 8	8 (49)
2	HCV	Core	131–140	ADLMGYIPLV	4, 8, 22	13 (75)
3	HCV	E1	257–266	QLRRHIDLLV	22	7 (51)
4	HCV	NS3	1073–1081	CINGVCWTV	8	16 (72)
5	HCV	NS3	1169–1177	LLCPAGHAV	8	17 (87)
6	HCV	NS3	1406–1415	KLVALGINAV	8	7 (42)
7	HCV	NS4B	1789–1797	SLMAFTAAV	4, 8, 22	16 (75)
8	HCV	NS4B	1807–1816	LLFNILGGWV	8	19 (124)
9	HCV	NS4B	1851–1859	ILAGYGAGV	4	14 (88)
10	HCV	NS5B	2727–2735	GLQDCTMLV	4	9 (41)
A	HBV	Core	18–27	FLPSDFFPSV	5	4 (29)
B	HBV	Env	183–191	FLLTRILTI	21	18 (59)
C	HBV	Env	335–343	WLSLLVPFV	17	14 (115)
D	HBV	Pol	455–463	GLSRYVARL	20	12 (81)
E	HBV	Pol	575–583	FLLSLGIHL	20	15 (119)

[a] HCV peptide sequences and termini are based on the HCV-1 isolate (genotype 1a) as described elsewhere (6). Percent sequence conservations are based on comparison with all HBV and HCV sequences available in the GenBank database.

TABLE 5. Characterization of patient subgroups based on HCV genotype[a]

HCV genotype	Mean total CRI	Positive CTL responses (%)	Mean HCV RNA[b]	Mean ALT (U/liter)	n[c]
1a	669 ± 322	36 (51/140)	79 ± 126	121 ± 67	14
1b	596 ± 503	34 (31/90)	65 ± 42	105 ± 103	9
2a	137 ± 174	0 (0/20)	18 ± 15	59 ± 13	2
2b	741 ± 393	36 (29/80)	15 ± 17	63 ± 34	8
3a	813 ± 308	45 (27/60)	20 ± 20	138 ± 145	6

[a] The values for total CRI and percentages of positive CTL responses in patients infected with HCV genotypes 1a, 1b, 2b, and 3a are statistically not significantly different ($P > 0.05$). These values are lower in patients infected with HCV genotype 2a ($P < 0.01$), but this difference may be due to the small number of patients.
[b] The difference in mean HCV RNA titers between genotype 1a versus 2b and 3a is statistically significant ($P < 0.01$), but not when they are corrected for the two to threefold lower sensitivity of the HCV bDNA assay for HCV genotypes 2b and 3a.
[c] n, number of samples for which CTL responsiveness, HCV RNA, and ALT values were determined.

Recombinant expression vectors. A recombinant vaccinia virus construct, vHCV827—3011, that encodes part of the HCV NS2 protein as well as the entire NS3, NS4A, NS4B, NS5A, and NS5B proteins according to the sequence of the HCV-1a isolate (9) under the control of the T7 promoter (9) was used together with a helper vaccinia virus expressing the T7 RNA polymerase (vT7) to induce transient expression of endogenously processed HCV proteins in human Epstein-Barr virus (EBV) B-cell lines (B-LCL).

Stimulation of PBMC with synthetic peptides. A multiple microwell in vitro peptide stimulation procedure was employed in these studies to expand HBV- and HCV-specific CTLs prior to cytotoxicity analysis. PBMC from patients and normal donors were separated on Ficoll-Histopaque density gradients (Sigma Chemical Co., St. Louis, Mo.) and cryopreserved in freezing medium containing 70% fetal calf serum (GIBCO Laboratories), 10% dimethyl sulfoxide (Sigma Chemical Co.) and 20% Hanks balanced salt solution (HBSS [GIBCO Laboratories]). Immediately prior to study, the PBMC were rapidly thawed; washed three times in HBSS; resuspended in RPMI 1640 (GIBCO Laboratories) supplemented with L-glutamine (2 mM), gentamycin (10 mM), and 10% heat-inactivated human AB serum; and plated in 96-well round-bottom plates at 0.4×10^6 cells per 100-μl well. Synthetic peptides were added at 10 μg/ml to eight replicate cultures. HBcAg was added at 1 μg/ml as a source of T-cell help (5, 17, 20) to PBMC from HBV-infected patients only. On day 7, the cultures were transferred into a 96-well flat-bottom plate, and on days 7 and 14 the cultures were restimulated with 10 μg of peptide and 20 U of recombinant interleukin-2 (Hoffmann-La Roche, Inc., Nutley, N.J.) per ml and 10^5 irradiated (3,000 rad) autologous feeder cells. On days 3, 10, and 18, 100 μl of RPMI 1640 with 10% (vol/vol) human AB serum and recombinant interleukin-2 at a 10-U/ml final concentration was added to each well. Cultures from the patients with acute HBV infection were tested for cytotoxic activity on day 14. All cultures from patients with chronic HBV or chronic HCV infection were tested on day 21. PBMC cultures from individual patients that displayed peptide-specific cytotoxicity against peptide-pulsed JY-EBV on day 21 were combined in one well of a 24-well plate in a total volume of 1 ml and restimulated with 10^6 irradiated (3,000 rad) autologous feeders, 10 μg of peptide per ml, and a final concentration of recombinant interleukin-2 of 10 U/ml, and the peptide-specific CTL lines were used to further define the characteristics of the CTL response.

Target cell lines. Autologous and allogeneic EBV-transformed B-LCL were established from our own pool of patients and normal donors as described elsewhere (5). For most studies, JY cell targets (HLA-A2.1, B7, and Cw7) were employed. All target cells were maintained in RPMI 1640 with 10% (vol/vol) heat-inactivated fetal calf serum (GIBCO Laboratories).

Cytotoxicity assay. Target cells consisted either of allogeneic, HLA-matched, and mismatched B-LCL incubated overnight with synthetic peptides at 10 μg/ml or of B-LCL infected with recombinant vaccinia viruses. Vaccinia virus-infected targets were prepared by infection of 10^6 B-LCL with vHCV827-3011 plus the helper vaccinia virus vT7 at a multiplicity of infection of 10 on a rocking plate at room temperature for 1 h and then by a single wash and overnight incubation at 37°C. B-LCL infected with vT7 alone were used as a control. Target cells were labeled with 100 μCi of ^{51}Cr (Amersham Corp., Arlington Heights, Ill.) for 1 h and washed four times with HBSS. Cytolytic activity was determined in a stan-

dard 4-h ^{51}Cr release assay by using round-bottom 96-well plates with 3,000 targets per well. Stimulated PBMC from patients and normal controls were tested at effector-to-target (E-to-T) ratios of 30:1 to 50:1. For each peptide, eight replicate cultures were tested. Percent cytotoxicity was determined by the formula $100 \times [(\text{experimental release} - \text{spontaneous release})/(\text{maximum release} - \text{spontaneous release})]$. Maximum release was determined by lysis of targets with 10% Triton X-100 (Sigma Chemical Co.).

CRI. As previously described (21), the analysis of eight replicate cultures for each peptide allowed the strength of the CTL response to be expressed as the CTL response index (CRI) for that peptide (CRI-P). The total CRI is the sum of all specific cytotoxicities of the eight replicate cultures for a given peptide in a patient. The total CRI value is the sum of all of the CRI-P values for each patient and provides a broader representation of the overall CTL response to the entire panel of epitopes in each patient. CRI-P values of more than 3 standard deviations (SD) above the mean CRI-P values observed in the uninfected control group were considered positive (Table 4).

HLA typing. HLA typing of PBMC from patients and from uninfected control subjects was performed by complement-dependent microcytotoxicity with HLA typing trays purchased from One Lambda (Los Angeles, Calif.).

HCV RNA determination. RNA was extracted from 100 μl of serum as previously described (11). Reverse transcription-nested PCR of the conserved 5′ noncoding region was performed to establish the presence of HCV RNA (9). PCR products were analyzed on a 1.5% agarose gel and were visualized by ethidium bromide staining. HCV RNA was quantitated by the branched-chain DNA (bDNA) nucleic acid hybridization Quantiplex HCV RNA assay (Chiron Corporation, Emeryville, Calif.) according to the manufacturer's instructions. The results were expressed as 10^5 HCV genome equivalents per ml. HCV RNA content in bDNA-negative samples was also estimated by semiquantitative PCR with serial 10-fold dilutions of cDNA and was expressed as the negative logarithm (base 10) of the highest 10-fold dilution retaining PCR positivity. The HCV genotype for each patient was determined by the standard INNOLIPA assay (National Genetics Institute, Los Angeles, Calif. [courtesy of Andrew Conrad]) by utilizing amplified viral nucleic acid and genotype-specific probes in the 5′ noncoding region (23).

Statistical analysis. The Wilcoxon signed rank test was used to compare ALT, total CRI, and HCV RNA values before versus during or after interferon therapy in the paired samples of patients HCV-13 to HCV-22 (see Fig. 7A to C). The Mann-Whitney nonparametric two-sample rank test was used to compare the same values in unpaired samples of patients HCV-1 to HCV-12 studied only before versus samples of patients HCV-23 to HCV-36 studied only during or after interferon therapy. The rank Kruskall-Wallis one-way nonparametric analysis of variance test was used to compare total CRI, ALT, and HCV RNA values in groups of patients infected with different HCV genotypes (Table 5) and to compare total CRI and ALT values in bDNA-positive and -negative samples (Table 6). Finally, standard chi-square tests and chi-square tests for trend were used to assess differences between the numbers of positive CTL assays in patients with different total CRI ranges (Table 7) in patients tested before and during or after interferon therapy and in patients infected with different HCV genotypes

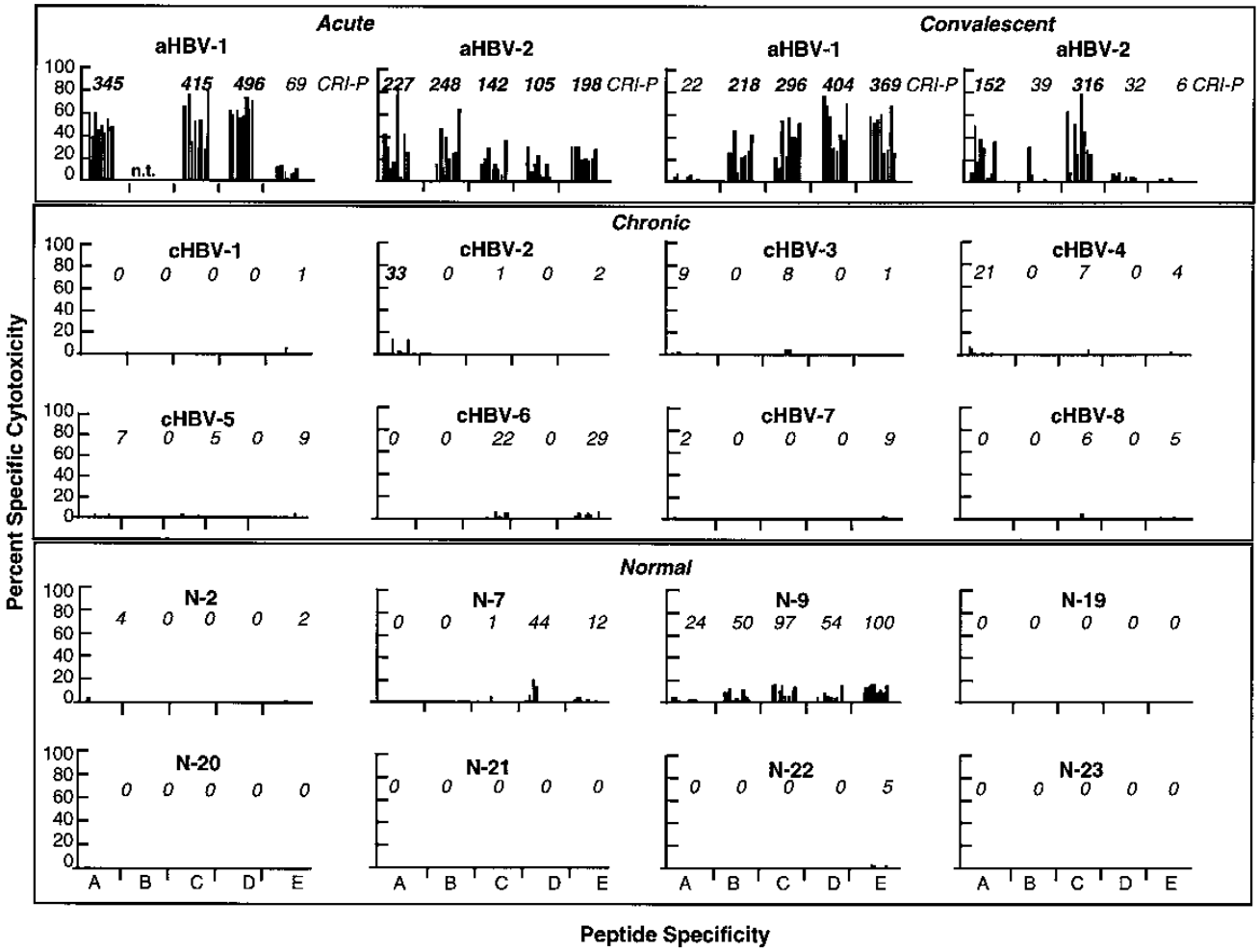

FIG. 1. HBV-specific CTL responses in patients with acute hepatitis B (aHBV-1 and aHBV-2) and chronic hepatitis B (cHBV-1 to cHBV-8) and healthy, uninfected controls (N-2, N-7, N-9, N-19, N-20, N-21, N-22, and N-23). Patients aHBV-1 and aHBV-2 were also tested after clinical and serological recovery, 21 and 19 months after the onset of acute hepatitis, respectively. At this time, both patients had normal ALT activity, had lost HBeAg and HBsAg, and were anti-HBeAg and anti-HBsAg positive. PBMC were stimulated with 10 μg of peptide per ml as described in Materials and Methods. For patients aHBV-1 and aHBV-2, the CTL assay was performed on day 14, while for patients cHBV1-8 and all uninfected controls, it was done on day 21. CTLs were tested in a 4-h ^{51}Cr release assay against JY target cells prepulsed overnight with the same peptide. The results shown are percentages of specific lysis in a 4-h ^{51}Cr release assay at an E-to-T-cell ratio of 30:1 to 40:1. CRI-P values are indicated; indices greater than or equal to 3 SD above the means for healthy, uninfected controls are indicated in boldface type. The peptide specificity and the cutoff for positivity are shown in Table 4. nt, not tested.

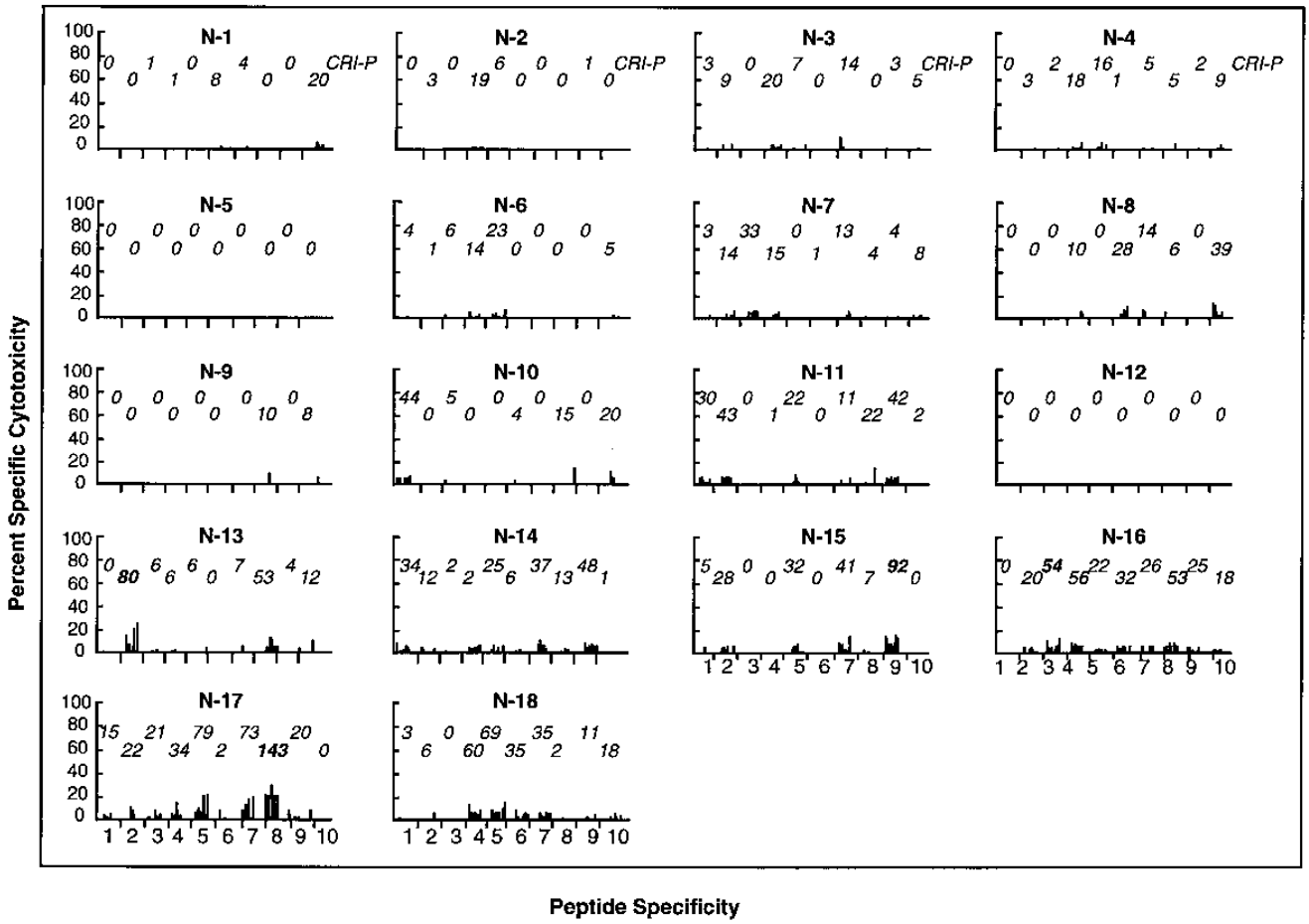

FIG. 2. HCV-specific CTL response in anti-HCV negative controls (N-1 to N-18). PBMC were stimulated with 10 μg of peptide per ml for 3 weeks as described in Materials and Methods and were tested in a 4-h ^{51}Cr release assay against JY target cells prepulsed overnight with the same peptide. The results shown are percentages of specific lysis in a 4-h ^{51}Cr release assay at an E-to-T-cell ratio of 30:1 to 40:1. CRI-P values are indicated; indices greater than or equal to 3 SD above the mean for healthy, uninfected controls are indicated in boldface type. The peptide specificity and the cutoff for positivity are shown in Table 4.

(Table 5). Differences with a two-sided P value of <0.05 were considered statistically significant.

RESULTS

Establishment of parameters defining a positive CTL response. To determine the normal range of cytolytic activity against the HBV and HCV peptides, we tested the CTL responses to these peptides in 23 normal uninfected controls (Table 3). As seen in Fig. 1 (HBV) and 2 (HCV), most of the normal controls were entirely nonresponsive in these assays. To compare the relative strengths of the CTL responses in patients and controls, CRI-P values were calculated as described in Materials and Methods and are shown above each set of peptide-specific responses in Fig. 1 to 4. The mean + 3 SD CRI-P values observed in the uninfected controls for each peptide are provided in Table 4. CRI-P values of greater than 3 SD above the mean control CRI-P value for each peptide were considered positive responses in this study. These values are indicated in boldface type in Fig. 1 to 4. The sum of all CRI-P values for each patient reflects the overall strength of the CTL response to all of the corresponding viral peptides and is designated the total CRI (see below).

Analysis of HBV-specific CTL in HBV-infected patients and uninfected controls. In keeping with our previous studies using a single macrowell peptide stimulation strategy (8, 20), the five HBV-derived epitopes were recognized vigorously by two patients who were studied by the multiple microwell stimulation technique during the acute and convalescent phases of HBV infection (Fig. 1, top). In contrast, only one (marginal) CTL response (patient cHBV-2 [peptide A; CRI-P, 33]) was detected in the peripheral blood of eight patients with chronic hepatitis B (Fig. 1, middle), and no responses were detected in the eight uninfected controls (Fig. 1, bottom), although one normal donor (N-9) displayed low-level reactivity to several peptides.

Analysis of the HCV-specific CTL response in HCV-infected patients and uninfected controls. In contrast to the nonresponsiveness of the chronic HBV patients (Fig. 1), 35 of the 36 patients (97%) chronically infected by HCV responded to at least one of the HCV epitopes, and many patients responded to many epitopes (Fig. 3 and 4). Specifically, 21 of the 22 patients who were studied before interferon therapy (patients HCV-1 to HCV-22 [Table 1; Fig. 3]) showed HCV-specific CTL responses. This produced a combined total of 102 of a possible 220 positive CTL responses (46%), with patient HCV-4 scoring negative by these criteria. Similarly, 19 of 24 patients (HCV-13 to HCV-36 [Table 1; Fig. 4]) studied during or after interferon therapy produced CTL responses to be-

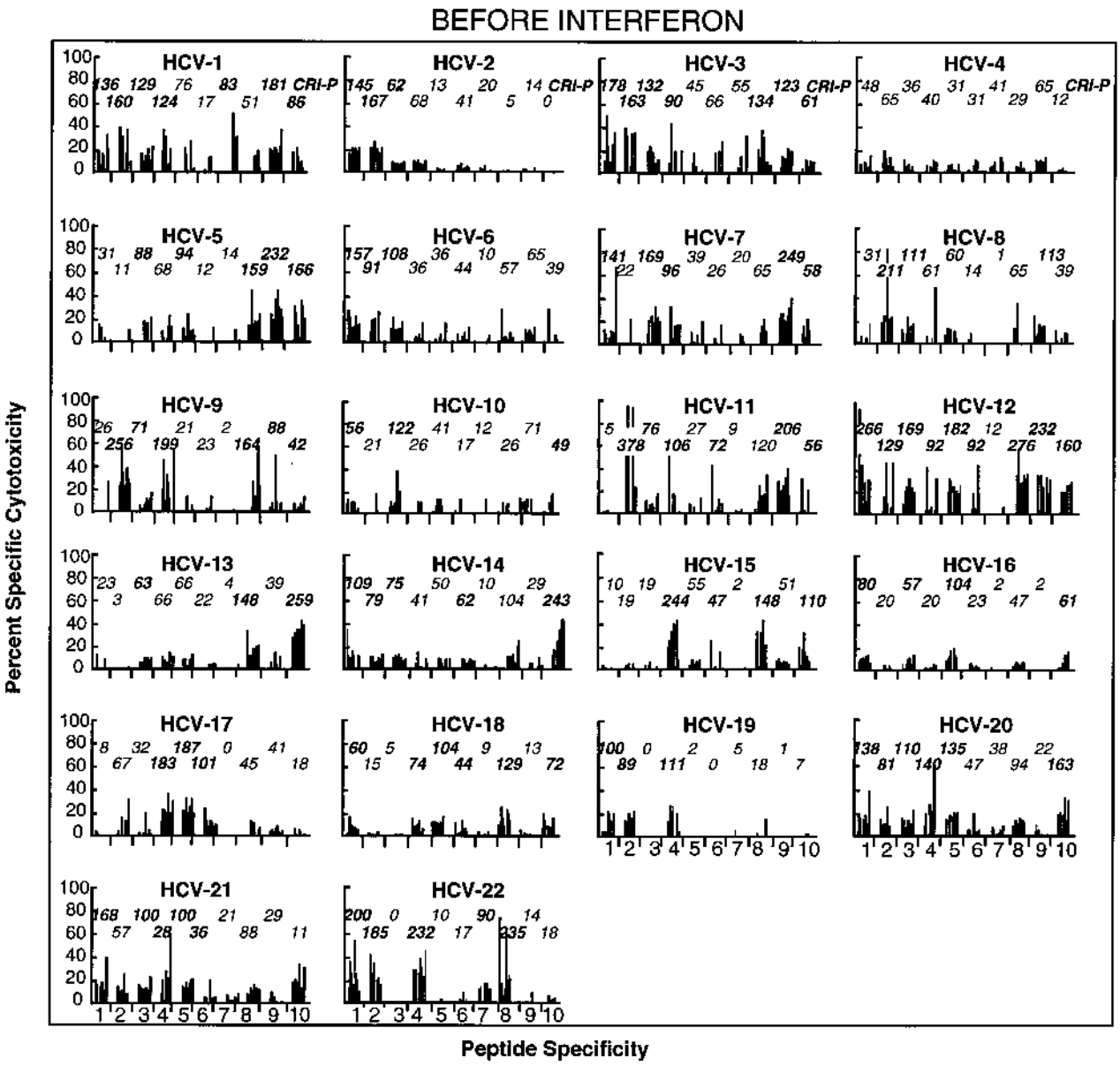

FIG. 3. HCV-specific CTL responses in untreated patients with chronic hepatitis C (HCV-1 to HCV-12 and HCV-13 to HCV-22). PBMC were stimulated with 10 μg of peptide per ml for 3 weeks as described in Materials and Methods and were tested in a 4-h ^{51}Cr release assay against JY target cells prepulsed overnight with the same peptide. The results shown are percentages of specific lysis in a 4-h ^{51}Cr release assay at an E-to-T-cell ratio of 30:1 to 40:1. CRI-P values are indicated above each peptide; CRI-P values greater than or equal to 3 SD above the mean for healthy, uninfected controls are indicated in boldface type. The peptide specificity and the cutoff for positivity are shown in Table 4.

tween one and six peptides (Fig. 4), yielding a total of 71 CTL responses of a possible 240 (30%). In contrast, only 4 of the 18 controls (Fig. 2) each produced a single marginal CTL response, yielding a combined total of 4 of a possible 180 positive CTL responses (2.2%). The basis for these 4 CTL responses is not clear, because only one of the 4 subjects (N-13) had an occupational risk of HCV infection and all 4 were confirmed to be negative for anti-HCV-antibodies and HCV RNA by reverse transcription-nested PCR (not shown). Nonetheless, these results must be considered in the context of the reported high prevalence of HCV infection in this country (1–3).

Functional characterization of HCV-specific CTL responses to previously uncharacterized epitopes. We have previously demonstrated that the CTL response to most of these HCV epitopes is restricted by the HLA-A2 allele and is mediated by $CD8^+$ T cells that recognize endogenously synthesized antigen (8). Since we have not previously documented these characteristics for the responses to peptides 5 (HCV1169-1177) and 10 (HCV2727-2735), we examined the corresponding peptide-specific CTL lines derived from patients HCV-17 and HCV-24 for their ability to recognize endogenously synthesized antigen. As shown in Fig. 5, peptide-specific lines from these 2 patients recognized target cells that were infected by vHCV827-3011 and synthesized the corresponding NS3 (Fig. 5A) and NS5 (Fig. 5B) proteins endogenously. Specific cytotoxicity was me-

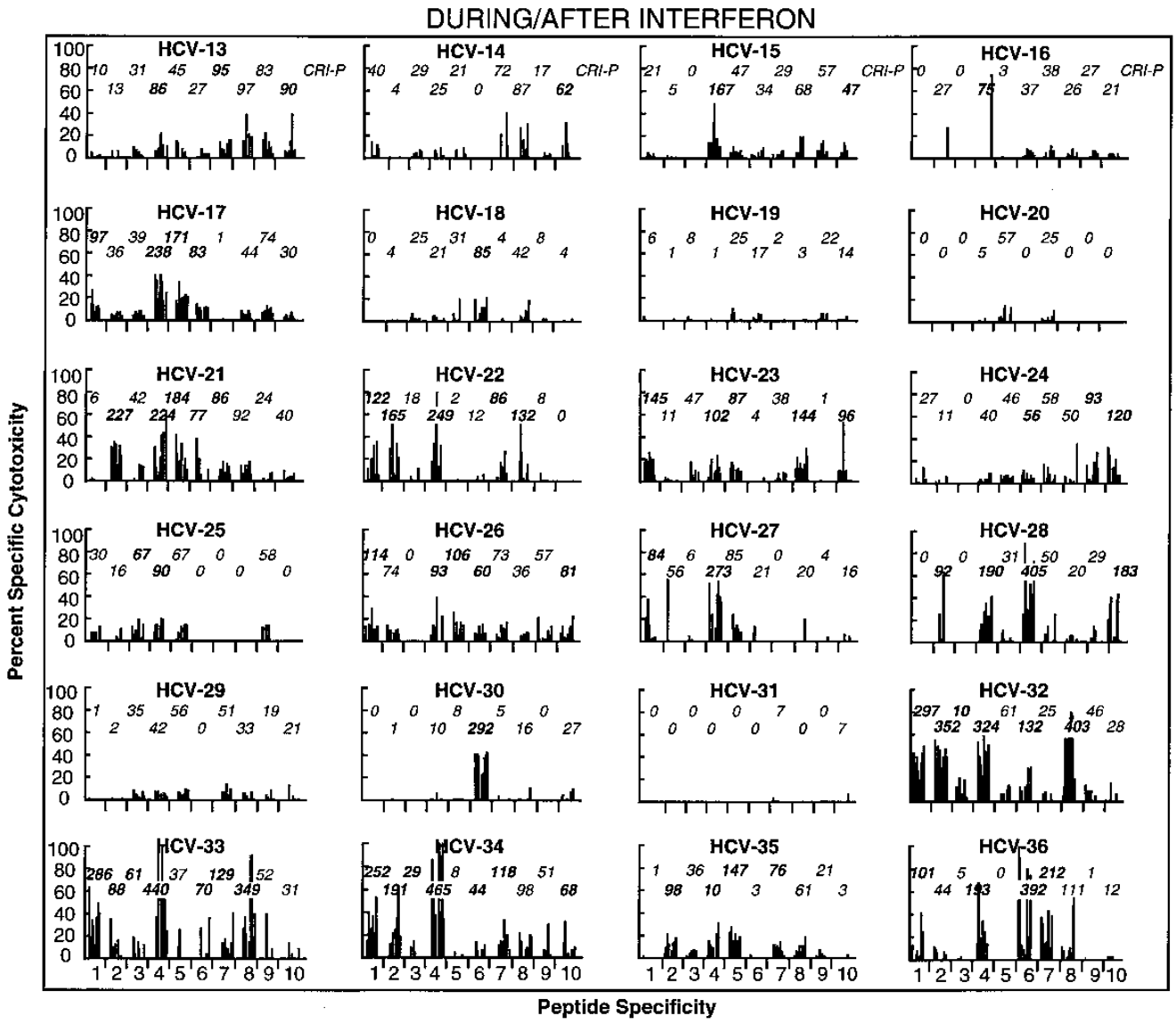

FIG. 4. HCV-specific CTL response in interferon-treated patients with chronic hepatitis C (HCV-13 to HCV-22 and HCV-23 to HCV-36). PBMC were stimulated with 10 μg of peptide per ml for 3 weeks as described in Materials and Methods and were tested in a 4-h ^{51}Cr release assay against JY target cells prepulsed overnight with the same peptide. The results shown are percentages of specific lysis in a 4-h ^{51}Cr release assay at an E-to-T-cell ratio of 30:1 to 40:1. CRI-P values are indicated; indices greater than or equal to 3 SD above the mean for healthy, uninfected controls are indicated in boldface type. The peptide specificity and the cutoff for positivity are shown in Table 4.

diated by $CD8^+$ cells, since it could be blocked with anti-CD8 but not with anti-CD4 antibodies (Fig. 5C and D). As expected, the CTL were HLA-A2 restricted, since both epitopes were recognized only when they were presented by heterologous B-LCL sharing only the HLA-A2 allele with the effector cells (Fig. 5E and F).

Influence of HCV genotype on CTL responsiveness. Interestingly, PBMC from patients infected with different HCV genotypes (Table 1) responded equally well to the peptides that we used in this study, despite the fact that the peptide sequences were derived from HCV genotype 1a. As shown in Table 5, patients infected by HCV genotype 1a produced CTL responses in 51 of 140 possible instances (36%). Similarly, patients infected by HCV genotypes 1b, 2b, and 3a displayed 31 of 90 CTL responses (34%), 29 of 80 CTL responses (36%), and 36 of 60 CTL responses (45%), respectively. The mean total CRI values were also not significantly different in patients infected by these HCV genotypes (Table 5). Both of these values were significantly lower in patients infected with HCV genotype 2a, but this may be due to the small number of patients ($n = 2$) infected with this genotype that were enrolled in this study. These results suggest that the peptides used in this study, while derived from the HCV-1 (genotype 1a) sequence, are broadly cross-reactive. They also suggest that differential CTL responsiveness to these particular peptides is not greatly influenced by the genotype of the infecting virus.

Comparison of CTL response with disease activity and viral load. To examine the relationship between CTL response and viral load, HCV RNA was quantitated by bDNA analysis, and bDNA-negative samples were analyzed by semiquantitative re-

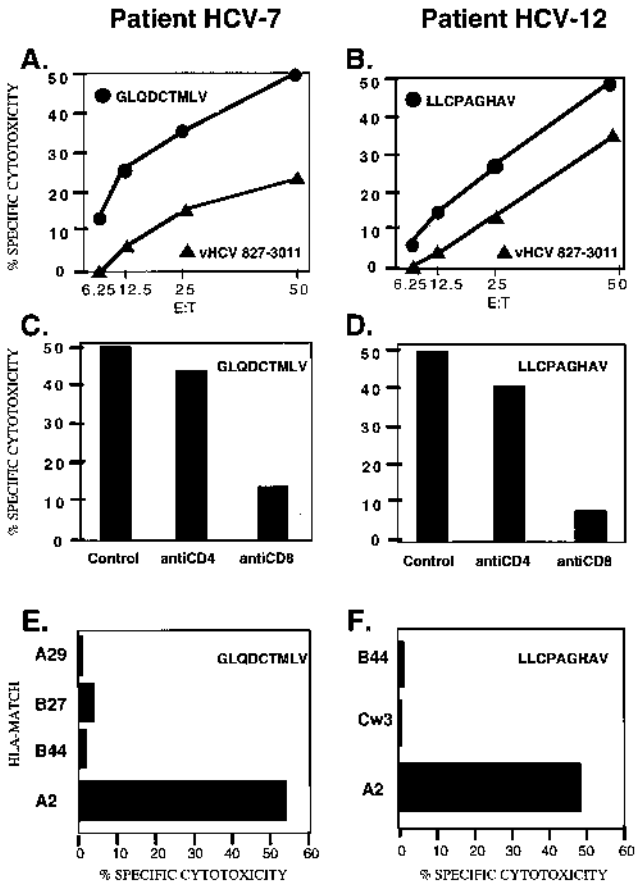

FIG. 5. (A and B) $CD8^+$ cells recognize endogenously synthesized antigen. Epitope-specific CTL lines were generated by stimulating PBMC from patients HCV-17 and HCV-24 with 10 μg of the indicated peptides per ml as described in Materials and Methods. Peptide-specific lines were tested for specific cytotoxicity against target cells (JY-EBV) either pulsed with 10 μg of peptide per ml (●) or infected with recombinant vHCV827-3011 together with the helper vaccinia virus vT7 (▲) in a standard 4-h ^{51}Cr release assay at various E-to-T-cell ratios. vT7 alone or JY-EBV targets without peptide were used as controls. (C and D) Anti-CD4 and anti-CD8 (Becton Dickinson, San Jose, Calif.) were added to the epitope-specific CTL lines described above at 10 μg/ml for 30 min before the addition of target cells. Epitope-specific lysis was determined at an E-to-T-cell ratio of 50:1. (E and F) Epitope-specific CTL lines were tested against allogeneic, partly HLA-matched B-LCL prepulsed overnight with 10 μg of peptide per ml. Cytotoxicity was measured at an E-to-T-cell ratio of 50:1 in a 4-h ^{51}Cr release assay.

verse transcription-PCR. Of all 44 serum samples analyzed, 31 (71%) were positive by bDNA analysis, i.e., contained more than 3.5×10^5 HCV RNA genome equivalents per ml (Table 1). Among the 13 serum samples that were negative by bDNA analysis, 8 were positive by PCR over a dilution range varying from 10^0 to 10^{-4}, and 5 were negative by PCR analysis (Table 1). As shown in Fig. 6, cross-sectional analysis of all assays performed failed to reveal an obvious correlation between total CRI and either HCV RNA levels (Fig. 6A) or ALT activity (Fig. 6B). Surprisingly, however, grouping of the assay results based on viral load or total CRI thresholds did reveal an inverse relationship between total CRI and serum HCV RNA content. As shown in Table 6, patients who were HCV RNA negative by bDNA analysis (i.e., $<3.5 \times 10^5$ HCV genome equivalents per ml) displayed somewhat higher mean total CRI values and lower mean ALT levels than patients who were HCV RNA positive by bDNA analysis. While this difference

TABLE 6. Characterization of patient subgroups based on HCV RNA detection by bDNA analysis

Subgroup	HCV RNA	Mean total CRI	Mean ALT (U/liter)	*n*
A	+	634 ± 365[a]	115 ± 89[b]	31
B	−	926 ± 392[a]	50 ± 18[b]	23
Control	−	126 ± 114		18

[a] *P* (A versus B), 0.0297.
[b] *P* (A versus B), 0.006.

was not very great, it was statistically significant ($P = 0.0297$ and $P = 0.006$ for differences in mean total CRI and ALT). Perhaps more convincingly, the percentage of patients (60%) with a total CRI of >1,000 who were HCV RNA negative by bDNA analysis was much higher than those of patients whose total CRIs were between 501 and 1,000 (29%) or were equal to or less than 500 (8%), as shown in Table 7. This trend of increasing frequency of bDNA negativity with increasing total CRI values was statistically significant, as was the difference between the two groups with the lowest and the highest total CRIs ($P = 0.02$). However, it is noteworthy that while there appears to be an inverse relationship between the strength of the CTL response and HCV bDNA positivity, the two patients who showed a sustained complete response to interferon (HCV-24 and HCV-26) did not display particularly high total CRI values (Table 1). Hence, the role of the CTL response in viral elimination in these patients is not clear.

Influence of interferon therapy on total CRI, disease activity, and viral load. Using the same total CRI and HCV RNA thresholds, the 22 assays from patients HCV-1 to HCV-22 tested before interferon therapy and the 24 assays from patients HCV-13 to HCV-36 tested during or after treatment displayed the same inverse association between total CRI and HCV RNA that we observed when the entire database was analyzed, suggesting that this relationship is independent of interferon (Table 7), although the numbers in both groups were too small to reach statistical significance.

Overall, both of these parameters as well as mean ALT levels decreased during interferon therapy, as shown in Fig. 7. Ten patients (HCV-13 to HCV-22 [Table 1]) were tested twice, both before and during or after interferon therapy, allowing analysis of 10 paired samples. As shown in Fig. 7A, the mean total CRI as a measure of the overall strength of the CTL response to all of the peptides decreased from 702 before interferon therapy to 468 after interferon therapy ($P = 0.019$). The same conclusion was evident at the level of the number of CTL responses. For example, before interferon therapy all 10 patients (100%) produced CTL responses to between three and six of the epitopes, yielding a combined total of 43 CTL responses of a possible 100 (43%). In contrast, after therapy, 8 of the 10 patients (80%) produced a total of 22 CTL responses

TABLE 7. Characterization of patient subgroups based on CRI

Subgroup	Total CRI range	bDNA-negative samples (%)		
		Total[a]	Before interferon therapy	During or after interferon therapy
A	14–500	8 (1/13)	0 (0/4)	11 (1/9)
B	501–1,000	29 (6/21)	15 (2/13)	50 (4/8)
C	1,001–1,269	60 (6/10)	40 (2/5)	80 (4/5)

[a] *P* (8% versus 60%), 0.02. Trend, $P = 0.0064$.

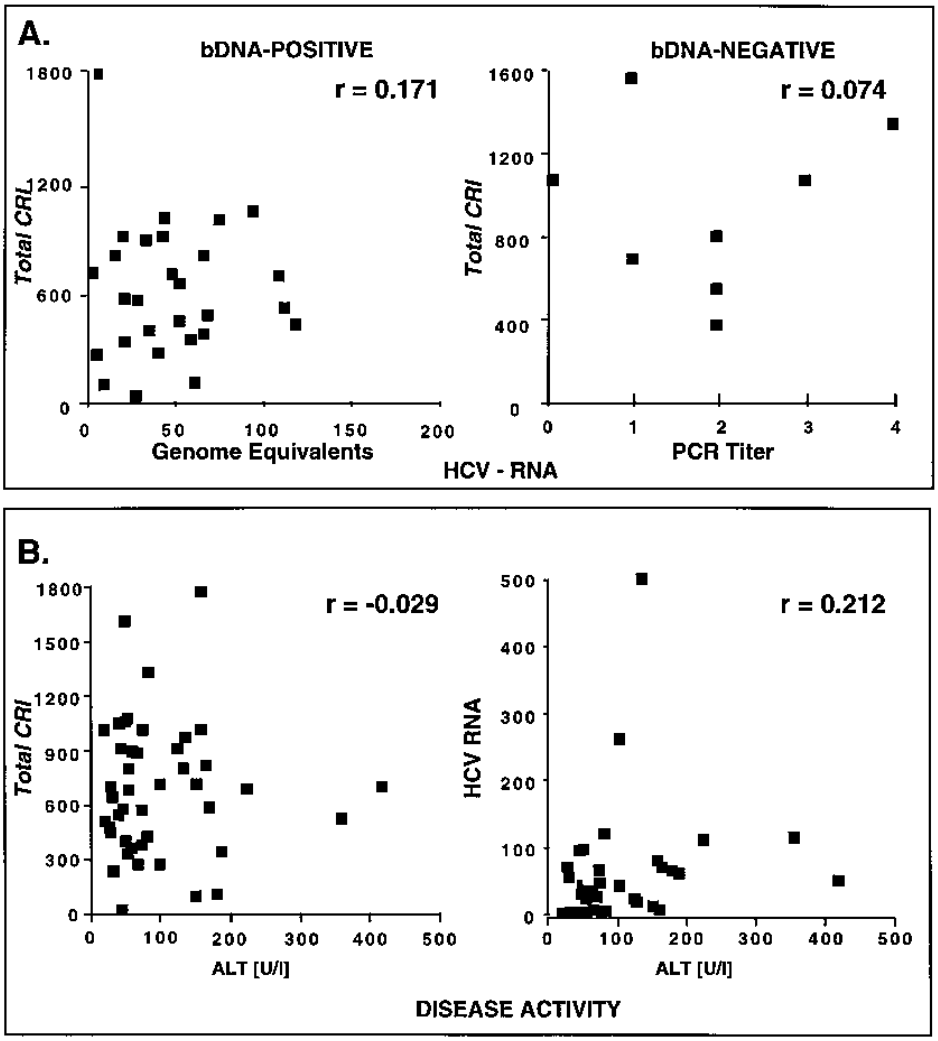

FIG. 6. (A) Cross-sectional analysis of data obtained from all the HCV-infected patients (HCV-1 to HCV-36) at all bleed dates, i.e., before and during or after interferon therapy. HCV RNA was measured by bDNA analysis (expressed as 10^5 HCV genome equivalents per ml of serum) or PCR (expressed as the highest 10-fold dilution of the cDNA yielding a positive signal). r, correlation coefficient. (B) Correlation between ALT activity and total CRI or HCV RNA content. HCV RNA is expressed either as the number of genome equivalents $\times$ 10^5 per ml of plasma (in the bDNA-positive samples). The data represent a cross-sectional analysis of all samples tested as shown in Table 1.

of a possible 100 (22%) (P = 0.002). Similarly, ALT values decreased significantly during interferon therapy, as shown in Fig. 7B (P = 0.032). Decreases in HCV RNA load were found in most patients, except for patient HCV-15, who was tested 1 year after interferon therapy, at which time his HCV RNA titers had already risen again (Fig. 7C).

To extend these observations, we also compared the HCV-specific CTL responses in the 12 patients studied only before therapy (HCV-1 to HCV-12 [Table 1]) with those of the 14 patients (HCV-23 to HCV-36) studied only during or after interferon therapy (Table 1). The analysis of the unpaired data from these two groups showed that the total CRI as well as the percentage of positive CRI-P responses decreased with interferon therapy (not shown). Similarly, a decrease in ALT aminotransferase activity as well as HCV RNA values was observed, although none of these parameters reached statistical significance. These results demonstrate that interferon does not enhance and may even decrease the HCV-specific CTL response, similarly to its effects on serum ALT activity and HCV RNA levels.

DISCUSSION

The peripheral blood CTL precursor frequency for many pathogenic human viruses is frequently too low to detect without in vitro expansion by antigen stimulation (6, 12, 20, 25). Using a macrowell peptide stimulation protocol in which PBMC were stimulated for 14 days with HBV-derived peptides containing the HLA-A2.1-binding motif, we previously demonstrated that HLA-A2-restricted CTLs specific for HBV can be selectively expanded in the peripheral blood of acutely infected HLA-A2-positive patients (5, 17, 20). In contrast, we could not detect a significant CTL response in patients who were persistently infected by HBV or in healthy uninfected controls, even after 21 days of in vitro stimulation. These results indicate that a polyclonal and multispecific CTL response to HBV correlates with clearance of this virus, suggesting that the lack of such response may be responsible for HBV persistence. However, using the same macrowell technique and a 21-day stimulation schedule, we have shown that many patients with chronic HCV infection, unlike patients with chronic HBV

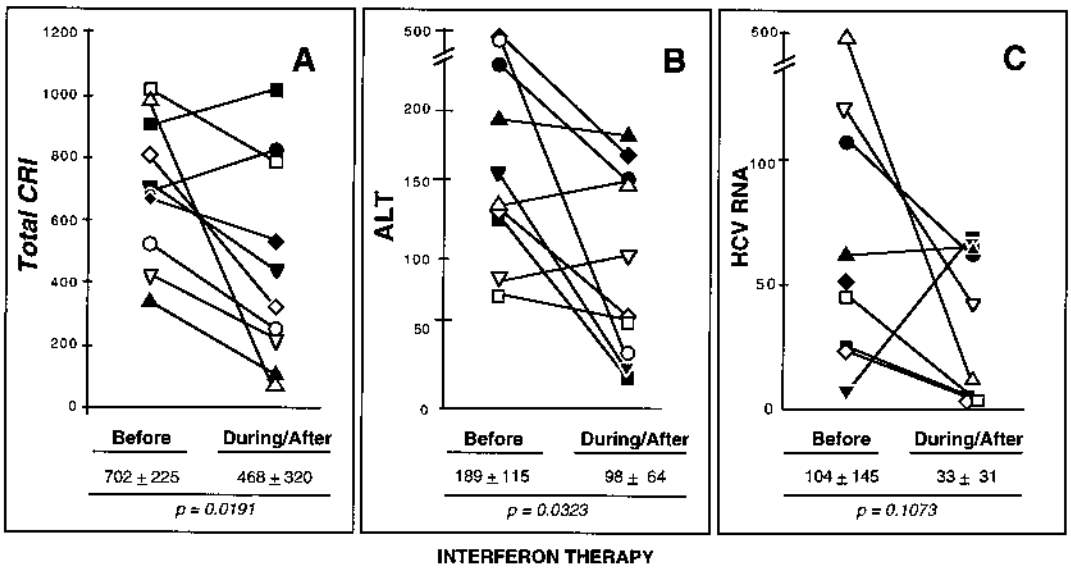

FIG. 7. Effect of interferon treatment on CRI, disease activity (ALT), and viral load (HCV RNA) as determined by analyzing patients HCV-13 to HCV-22 before and during or after interferon therapy. The total CRIs were determined as described in Materials and Methods. Serum ALT activities are given in units per liter, and HCV RNA values are given as numbers of genome equivalents $\times 10^5$ per milliliter serum. The patients (HCV-13 to HCV-22) are identified by the following symbols: ◆, HCV-13; ◇, HCV-14; ▼, HCV-15; ▽, HCV-16; ●, HCV-17; ○, HCV-18; ▲, HCV-19; △, HCV-20; ■, HCV-21; and □, HCV-22. The numbers at the bottom of each graph are the means ± SD of the total CRI, ALT, and HCV RNA values before and during or after interferon therapy.

infection, produce a peripheral blood CTL response to HCV-derived peptides (8) and that the CTL precursors express CD45RO, indicating that they were generated in vivo (8).

In the current study, we used a multiple microwell CTL stimulation protocol so that the strength of the CTL response to a single peptide or to the sum of all of the peptides could be more quantitatively compared with disease activity and viral load in these patients. Using this technique, we detected a CTL response to at least one of the HCV epitopes in the majority of HCV-infected patients (Fig. 3 and 4), and we showed that the CTL response is mediated by $CD8^+$, HLA-A2-restricted T cells that can recognize endogenously synthesized viral antigens (Fig. 5). In contrast, a marginal CTL response was detectable only rarely, and at low levels, in HCV-uninfected controls by this technique (Fig. 2).

Using the total CRI as a measure of the overall strength of the CTL response to HCV, we could not detect a positive or negative linear correlation between the CTL response and viral load in this population of patients with chronic HCV infection (Fig. 6). However, using a threshold analysis, we were able to show that a higher total CRI was associated with lower viral load, i.e., HCV RNA levels undetectable by bDNA analysis (Table 7). Although this correlation is far less satisfying than a linear relationship, it does suggest that the CTL response was able to exert some degree of control over viral load in chronically infected patients and that this relationship appeared to be independent of interferon therapy. The absence of a linear correlation between these two parameters, however, suggests that other variables probably affect CTL responsiveness and/or viral load in these patients. Of course, the CTL and/or the HCV assays themselves may not be linear. However, we have also failed to observe a linear correlation between HCV RNA levels and HCV-specific CTL precursor frequency in these patients by limiting dilution analysis (19), a well-accepted quantitative parameter of the strength of a CTL response (7). In the absence of other explanations, it is possible that viral load is influenced by other factors that we have not monitored in this study, including the class II-restricted helper T-cell response. Elucidation of this important possibility requires quantitative analysis of this other limb of the immune response in these patients.

Of course, the possibilities of viral sequence variation (24), infection of immune privileged sites, and interference with the antigen-processing machinery of the cell also represent viral strategies to escape the immune response that might be operative during HCV infection. However, further analysis is needed to evaluate these alternative scenarios. As far as viral sequence variation is concerned, we found that patients infected with different HCV genotypes recognize peptides derived from the HCV-1 sequence equally well. Since most of the epitope sequences in different genotypes contain conservative amino acid substitutions, broad cross-recognition may exist between the genotypes. Understanding the role of viral sequence variation in CTL responsiveness in individual patients will require immunological and viral sequence analysis at the clonal level, and these studies are currently in progress in our laboratory.

It is notable that in this study a strong CTL response was also associated with a low level of ALT activity (Table 6). This raised the possibility that HCV-specific CTLs exert their antiviral effect by noncytolytic mechanisms. Along this line, we have recently reported that HBV-specific CTLs can control HBV replication by secreting inflammatory cytokines that inhibit HBV gene expression and replication noncytolytically (13, 14). The inverse correlation between CTL responsiveness and disease activity may, therefore, suggest that HCV is partially responsive to noncytolytic control.

These observations, albeit not definitive, suggest that appropriate enhancement of the immune response to HCV may reduce viral load and possibly lead to viral clearance in chronically infected patients. Since the data also suggest that interferon therapy does not enhance the CTL response to HCV and may even reduce it (Fig. 7), perhaps by reducing viral load (i.e., antigenic stimulus) by more direct antiviral mechanisms, anti-

gen-specific immunotherapy may play a nonredundant role if it is added to interferon for the treatment of chronic HCV infection.

Prospective studies of acutely infected patients who clear HCV must now be performed to fully define the relationships between the breadth and strength of the CTL response, viral clearance, and disease activity in this infection.

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