HLA-A31- and HLA-Aw68-restricted Cytotoxic T Cell Responses to a Single Hepatitis B Virus Nucleocapsid Epitope during Acute Viral Hepatitis

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Summary

We have recently developed the technology to identify and characterize the human histocompatibility leukocyte antigen (HLA) class I–restricted, $CD8^+$ cytotoxic T lymphocyte (CTL) response to hepatitis B virus (HBV)-encoded antigens in patients with acute viral hepatitis. CTL are expanded in vitro by stimulation with HBV-derived synthetic peptides and selected by restimulation with a panel of HLA-matched stable transfectants that express the corresponding HBV protein. We have recently reported the existence of an HLA-A2-restricted, $CD8^+$ CTL response to an epitope located between residues 18 and 27 of the HBV nucleocapsid core antigen (HBcAg). We now report the discovery of a CTL epitope located between HBcAg residues 141 and 151 that completely overlaps a critical domain in the viral nucleocapsid protein that is essential for its nuclear localization and genome packaging functions as well as processing of the precore protein. The CTL response to this epitope is dually restricted by the HLA-A31 and HLA-Aw68 alleles, which, unexpectedly, appear to use a common binding motif based on the results of alanine substitution and competition analysis, and the binding properties of these two alleles predicted from their known primary sequence, and from the three-dimensional structure of HLA-Aw68. We have also demonstrated that the HBV-specific CTL response to this epitope is polyclonal during acute viral hepatitis, since these two restriction elements can present the HBcAg 141–151 epitope to independent CTL clones derived from a single patient; and that the CTL response is multispecific, since HLA-A2-restricted and HLA-Aw68-restricted CTL responses to HBcAg 18–27 and HBcAg 141–151, respectively, have been identified to coexist in another patient. The foregoing argue against the emergence of CTL escape mutants as a significant problem during HBV infection, especially at this locus, where mutations might be incompatible with viral replication. Finally, our data suggest an association between the HBV-specific CTL response and viral clearance, and they have implications for the design of immunotherapeutic strategies to terminate HBV infection in chronically infected patients.

The hepatitis B virus (HBV)[1] is a small hepatotropic enveloped virus with a double-stranded DNA genome (1) that causes acute and chronic necroinflammatory liver disease and hepatocellular carcinoma (2). The mechanisms responsible for viral clearance and liver cell injury in HBV infection are not well understood. Based on precedent in other viral systems, and a limited analysis of the intrahepatic (3–5) and PBL (6) response to HBV-encoded antigens, it appears that HBV-specific helper and CTL responses may play an important pathogenetic role in this disease. Since the viral nucleocapsid protein is an important target of the CTL response to other viruses (7–11), we have begun to examine the HLA class I–restricted CTL response to the hepatitis B nucleocapsid core antigen (HBcASg) in patients with acute and chronic viral hepatitis, type B.

In a recent series of studies, we demonstrated that >90%

[1] *Abbreviations used in this paper:* HBcAg, hepatitis B core antigen; HBV, hepatitis B virus.

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of HLA-A2-positive patients with acute HBV infection produce an HLA-A2-restricted, CD8-positive CTL response to a 10-residue epitope that maps between amino acids 18 and 27 (FLPSDFFPSV) of HBcAg (12). In contrast, we have shown that this response is not detectable in the peripheral blood in HLA-A2-positive patients with chronic hepatitis (13), suggesting that a vigorous CTL response to this epitope may contribute to viral clearance during HBV infection.

In the current study, we report the discovery and characterization of a new CTL epitope, recognized by patients with acute viral hepatitis, that maps to amino acids 141–151 of HBcAg (STLPETTVVRR). CTL recognition of this epitope is restricted by two independent class I molecules, HLA-A31 and HLA-Aw68, and both responses are focused on precisely the same 11-residue sequence. To our knowledge this represents the first report that two independent human class I molecules can present precisely the same endogenously synthesized epitope to independently rearranged TCRs. Furthermore, our data provide insight into the probable antigenic peptide binding motifs for these two independent HLA alleles. Additionally, together with our previous reports (12–14), the current observations suggest that the HLA class I–restricted CTL response plays an important role in viral clearance during acute HBV infection, and they raise the possibility that specific augmentation of this response might lead to viral clearance in patients with chronic hepatitis.

Materials and Methods

Patient Population. Six patients, five male and one female, with acute hepatitis B and nine normal blood donors were studied (Table 1). The diagnosis of acute hepatitis B was based on standard diagnostic criteria including clinical and biochemical evidence of severe liver cell injury with alanine aminotransferase (ALT) activity at least 20-fold higher than the upper limits of normal, together with serological evidence of acute HBV infection, including hepatitis B surface antigen (HBsAg) and IgM anti-HBc antibody (IgM HBc-Ab), and the absence of serological evidence of infection by the hepatitis δ or hepatitis C viruses (using commercially available reagents obtained from Abbot Laboratories, North Chicago, IL). All patients recovered completely from the illness, with normalization of serum transaminase and clearance of HBsAg within 4 mo of initial diagnosis.

Synthetic Peptides, HBV Antigens, and Tetanus Toxoid. A panel of overlapping synthetic peptides, 10–20 residues long, corresponding to the complete HBV core protein (subtype ayw), were purchased from Multiple Peptide Systems (San Diego, CA) or provided by Cytel Corporation (San Diego, CA). Peptides representing amino- and carboxy-terminal truncations of HBcAg 140–155, and a panel of peptides containing alanine substitutions scanning HBcAg 141–151, were produced by Chiron Mimotopes (Clayton, Australia). Lyophilized peptides were reconstituted at 10 mg/ml in sterile distilled water with or without 10% DMSO (Malinckrodt, Paris, KY). Recombinant (r)HBcAg was obtained from bacterial extracts of *Escherichia coli* as previously described (15). Tetanus toxoid protein was purchased from Connaught Laboratories (Swiftwater, PA).

Recombinant Expression Vectors. Recombinant vaccinia viruses expressing the HBV core (c-vac) region (ayw subtype) and the control wild-type vaccinia have been previously described (16). Another construct (WTe) that produces the HBV precore protein (H. J. Schlicht, unpublished observations) was also used. Stable transfectants that express HBcAg (ayw subtype) were produced by transfection of EBV-transformed B lymphoblastoid cell lines (B-LCL) with a panel of EBV-based expressed vectors that contain the HBV core region as previously described (17).

Stimulation of PBMC with Synthetic Peptides and rHBc. PBMC from patients and normal donors were separated on Ficoll-Hypaque density gradients (Sigma Chemical Co., St. Louis, MO), washed three times in HBSS (Gibco Laboratories, Grand Island, NY), resuspended in RPMI 1640 (Gibco Laboratories) supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), and Hepes (10 mM) containing 10% heat-inactivated human AB serum, and plated in a 24-well plate at 4×10^6 cells/well. The synthetic peptides, each at 10 μg/ml, were added to the cell cultures as follows: mixture 1 (core residues 1–20, 20–34, 28–47, 50–69, 70–89, 61–80); mixture 2 (core residues 82–101, 100–119, 120–139, 140–155, 155–169, 169–183); mixture 3 (precore residue 20–core residue 2, core residues 50–59, 117–131, 131–145, 111–125); mixture 4 (core residues 11–27, 91–110, 147–160, 162–176). rHBcAg (Biogen, Geneva, Switzerland) was added at 1 μg/ml to derive the benefit of a Th cell response within the culture during the first week of stimulation. At day 3, 1 ml of RPMI with 10% human AB serum and rIL-2 (Hoffman-La Roche, Inc., Nutley, NY) at 10 U/ml final concentration was added in each well, and the cultured PBMC were tested for CTL activity on day 7. PBMC that displayed cytolytic activity specific for one of the peptide mixtures used during the first week of stimulation were expanded by restimulation as described below.

Generation of HBV Core-specific CTL Lines. Generation of CTL line H1 from patient H.P., initially cultured with peptide mixture 2 plus rHBcAg, was performed by weekly restimulation with 5×10^5 autologous PBMC irradiated (3,000 rad) in RPMI plus 10% human AB serum, rIL-2 (20 U/ml), and peptide mixture 2 (first restimulation), or with peptide 140–155 (10 μg/ml) for all subsequent stimulations. CTL line E4 (from patient E.W.) and the CTL lines from four additional patients (V.T., H.F., Q.M., C.N.) were established by stimulating the PBMC with peptide 140–155 plus rHBcAg for the first week, with weekly restimulation thereafter with peptide 140–155 and rIL-2. The PBMC of the normal uninfected controls were stimulated similarly except that in selected instances tetanus toxoid was substituted for rHBc during the first week of stimulation to provide an alternate source of T cell help, since these individuals had not been previously exposed to HBcAg.

Generation of HBV-specific CTL Clones. CTL clones were generated by limiting dilution at one cell/well in 96-well microtiter plates from an HBV-specific CTL line, E4 (patient E.W.). After depletion of $CD4^+$ T cells from the CTL line by incubation with a CD4-specific mAb (Becton Dickinson & Co., Mountain View, CA) plus complement, the cells were plated in the presence of PHA (Sigma Chemical Co.) at 1 μg/ml, CD3-specific mAb at 0.5 μg/ml (Coulter Immunology, Hialeah, FL), rIL-2 (20 U/ml), and irradiated (5,000 rad) allogeneic PMBC (10^5/well). HBV specific clones were restimulated in a 24-well plate with 10^5 irradiated (9,000 rad) autologous transfectants expressing the HBV core region (described above), with 2×10^6 allogeneic irradiated (3,000 rad) PBMC feeder cells per well, in RPMI 1640 containing 10% heat-inactivated FCS and IL-2 (20 U/ml).

Target Cell Lines. Autologous and allogeneic EBV-transformed B-LCL were either purchased from The American Society for Histocompatibility and Immunogenetics (A.S.H.I.; Boston, MA) or established from our own pool of patients and normal donors as described (18). Line LB, HLA-Aw68.1$^+$ (described in reference

19), provided by Dr. D. C. Wiley, and line RSH (described in reference 19, purchased from A.S.H.I., were used in selected experiments. The cells were maintained in RPMI with 10% (vol/vol) heat-inactivated FCS (Gibco Laboratories). Short-term lines of autologous PBMC blasts were produced by stimulating PBMC with PHA at 1 μg/ml in RPMI with 10% FCS, 10 U/ml rIL-2 for 7 d before use as target cells (see below).

Cytotoxicity Assay. Target cells consisted either of: (*a*) autologous PHA-stimulated blasts or allogeneic HLA-matched and -mismatched B-LCL incubated overnight with synthetic peptides at varying concentrations between 0.001 and 10 μg/ml; (*b*) stable B-LCL transfectants described above; or (*c*) B-LCL infected with recombinant vaccinia viruses. In selected experiments (see Results) the target cells were labeled with ^{51}Cr for 1 h, followed by 1 h of incubation with synthetic peptides before the addition of effector cells. 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 three 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 between 60 and 100:1, whereas HBV core-specific CTL lines were tested at E:T ratios between 4 and 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 <25% of maximal release in all assays.

Flow Cytometry Analysis. Cells to be analyzed (0.5–1.0 × 10^6 cells) were washed once in PBS and then incubated with a fluorescent probe–conjugated anti-CD4 and anti-CD8 mAb (Leu3a, Leu2a) and similarly labeled control antibody (Becton Dickinson & Co.). After a 30-min incubation at 4°C, cells were washed in PBS with 5% BSA and 0.1% sodium azide, and analyzed with a FACScan® flow cytometer (Becton Dickinson & Co.).

HLA Typing. HLA typing of PBMC from patients and from normal donors was performed by microcytotoxicity using HLA typing trays purchased from One Lambda (Los Angeles, CA). The HLA haplotypes of six patients and nine uninfected normal controls used in this study are shown in Table 1.

Table 1. *HLA Class I Haplotype of the HBV-infected Patients, and the HLA-A31- and Aw68-positive Normal Donors Included in This Study*

Patient		HLA class I
HBV patient	E.W.	A31,Aw68,B35,Cw3,Cw4
	H.P.	A2,Aw68,B35,Bw62,Cw3,Cw4
	V.T.	A25,A31,B7,B18
	H.F.	A31,Aw68,Bw61,Cw3
	Q.M.	Aw36,Aw68,B49,Bw62,Cw1
	V.P.	A24,Aw68,B35,Bw67
	C.N.	A24,Aw68,Bw60,Cw3
Normal donor	a	A1,A31,B17,Bw60
	b	A2,A31,B27,B44,Cw1
	c	A3,A31,B7,B27
	d	A24,A31,B14,B35,Cw4
	e	A3,A31,B7
	f	A31,Aw68,B35,Bw60
	g	A3,Aw68,B7,B44,Cw7
	h	A1,Aw68,B8,B38,Cw7
	i	A11,Aw68,B35,B44,Cw4

Results

CTL Activity of PBMC Stimulated with Peptide Mixtures. PBMC from two patients (E.W., H.P.) with acute HBV infection were stimulated for 7 d with peptide mixtures, and then tested for cytolytic activity against autologous ^{51}Cr-labeled, PHA-activated blasts prepulsed with the same peptide mixture or with media. Interestingly, responses were observed to mixture 2 in both patients (Fig. 1). Synthetic peptides contained in mixture 2 represent HBcAg sequences that have not been previously described as recognition sites for HBV-specific human CTL. Accordingly, the remaining cells were restimulated for a second week with peptide mixture 2, and the antigenic specificity of the restimulated CTL line was established with autologous PHA-activated blast targets prepulsed with the individual peptides contained within the mixture. By this approach, peptide HBcAg 140–155 was shown to be responsible for the CTL activity induced by mixture 2 for both of these patients (Fig. 2). No cytotoxic activity was observed using unstimulated PBMC of patient E.W. as effectors against autologous B-LCL fed with peptide HBcAg 140–155 (data not shown), suggesting that specific CTL are present at low frequency in the peripheral blood during acute HBV infection. It is noteworthy that patient H.P. also displayed a CTL response to peptide mixture 4, and that pa-

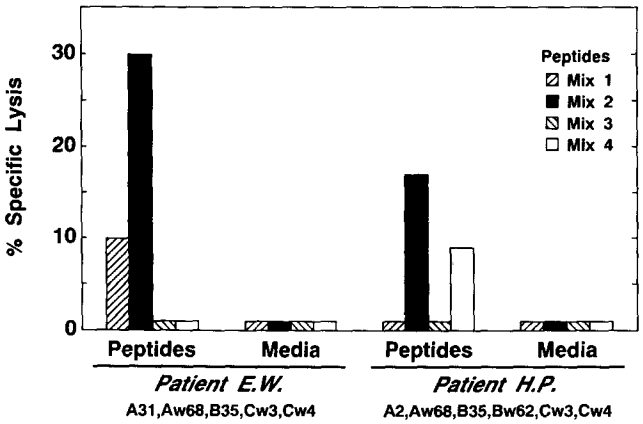

Figure 1. Activation of HBV-specific CTL by PBMC stimulation with mixtures of synthetic peptides. PBMC were stimulated for 1 wk with mixtures of HBcAg peptides and tested for cytotoxic activity in a 4-h ^{51}Cr release assay against autologous targets prepulsed with the same stimulatory mixture or with media only. The E/T ratio used was 70:1 for patient E.W. and 100:1 for patient H.P.

tient E.W. displayed a CTL response to mixture 1 (Fig. 1). Although the response to mixture 1 was transient and could not be further characterized, the response to mixture 4 was ultimately shown to be specific for core residues 18–27 and to be restricted by HLA-A2 (not shown), as we have previously reported (12–14, 17), demonstrating that multiple, independently restricted CTL responses to nonoverlapping CTL epitopes present on the same viral protein are readily detectable during acute HBV infection.

Generation and HLA Restriction Analysis of HBcAg 140–155-specific CTL Lines and Clones. HBcAg 140–155-specific CTL lines were generated by weekly stimulation of PBMC either with mixture 2 or with the active constituent peptide (core residues 140–155). Line E4 (patient E.W.) was started in the presence of rHBcAg and peptide HBcAg 140–155; line H1 (patient H.P.) was started in the presence of rHBc and mixture 2.

After 4 wk of restimulation, the HLA class I restriction of CTL line H1 was tested by using several allogeneic B-LCL target cells that were partially matched with the effector cells at the HLA class I loci but were completely HLA class II mismatched. The results, shown in Fig. 3, illustrate that the CTL activity was HLA-Aw68 restricted. The HBcAg 140–155-specific CTL line E4 was cloned at one cell per well in the presence of anti-CD3, PHA, and allogeneic PBL as feeder cells. After 2–3 wk, 15% of the seeded wells showed proliferation, and the growing cell populations were tested for specific lysis of autologous B-LCL preincubated with peptide core 140–155. Two clones (3D11, 2D7) that displayed highly efficient specific cytotoxic activity were selected for further analysis. The clones were tested against autologous and allogeneic B-LCL target cells partially matched with the effectors at the level of HLA class I and II alleles. The cytolytic activity of clone 3D11 was found to be HLA-A31 restricted, and the cytolytic activity of clone 2D7, derived from the same patient, was HLA-Aw68 restricted (Fig. 4). Both clones displayed the $CD4^-$, $CD8^+$ phenotype by flow cytometry (not shown).

These observations were confirmed and extended by analysis of four additional HLA-A31- or Aw68-positive patients

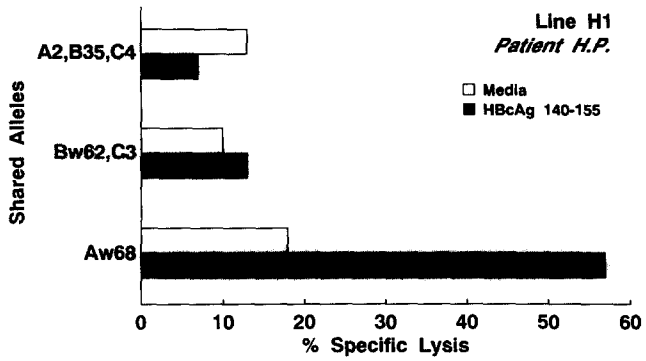

Figure 3. HLA restriction of the HBcAg 140–155-specific CTL response in patient H.P. A polyclonal CTL line (H1) specific for HBcAg 140–155 was incubated with ^{51}Cr-labeled B-LCL target cells that were prepulsed with peptide HBcAg 140–155 or with media. All target cells were completely mismatched at the level of class II and partially matched at the level of class I. The E/T ratio used was 4:1 in a 4-h ^{51}Cr release assay.

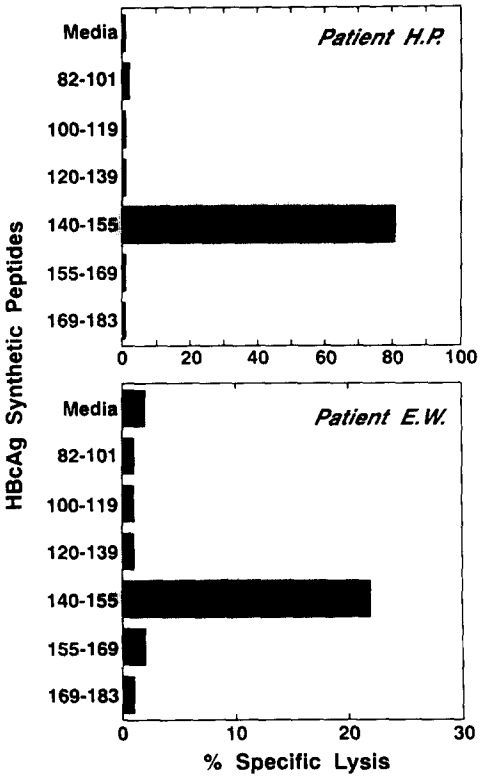

Figure 2. Identification of the target peptide in mixture 2. PBMC, stimulated by peptide mixture 2 for the first week, were restimulated with the same mixture of peptides and tested for cytotoxic activity in a 4-h ^{51}Cr release assay against ^{51}Cr-labeled autologous target cells prepulsed with the individual peptides in the mixture. The E/T ratio used was 50:1 for patient E.W. and 40:1 for patient H.P.

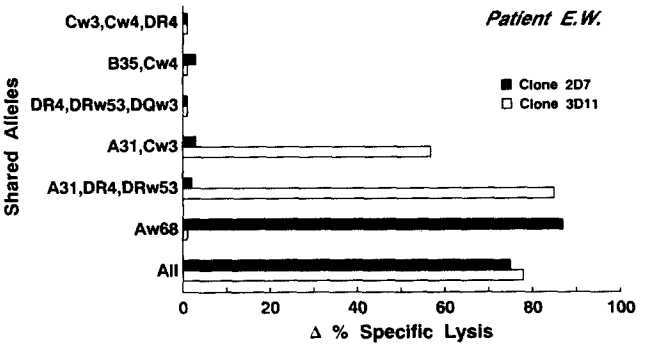

Figure 4. HLA restriction of the HBcAg 140–155-specific CTL response in patient E.W. Two HBcAg 140–155-specific CTL clones (3D11 and 2D7) were established by limiting dilution and incubated with ^{51}Cr-labeled autologous or allogeneic B-LCL target cells prepulsed with peptide 140–155 or media. Allogeneic target cells were matched at the level of class I and class II with the effector cells. The E/T ratio used was 10:1 in a 4-h ^{51}Cr release assay. Results are expressed as the difference between the percent ^{51}Cr release observed with peptide-pulsed targets and the percent ^{51}Cr release with media-pulsed target cells (which was <3% in all cases).

with acute HBV infection (H.F., V.T., Q.M., C.N.). In all these patients, HBcAg 140–155-specific CTL lines were generated as described for line E4. Using partially HLA-matched allogeneic target cells, the CTL response was shown to be restricted by the HLA-A31 allele in patient V.T.; it was clearly HLA-Aw68 restricted in patient Q.M. and probably Aw68 restricted in patient C.N. (Table 2). The response in patient H.F. was too weak to permit further analysis.

HBcAg 140–155-specific CTL Lines and Clones Can Lyse Target Cells That Express Endogenously Synthesized HBcAg. Two polyclonal CTL lines (E4, H1) and two clones derived from line E4 (3D11, 2D7) were tested for the ability to recognize endogenously synthesized nucleocapsid antigen by using autologous and allogeneic target cells that had been infected with recombinant vaccinia viruses that direct the synthesis of the HBV core and precore proteins by the cell. Line H1 (patient H.P.) and line E4 (patient E.W.) were tested against endogenously synthesized core protein induced by a recombinant vaccinia virus designated core-vac (Fig. 5). Clones 3D11 and 2D7 were tested against endogenously synthesized core and precore proteins induced by a recombinant vaccinia virus, designated core-vac and WTe-vac, respectively (Fig. 6). Significant levels of specific cytolytic activity were detected in all cases (Figs. 5 and 6). Recognition of endogenously synthesized antigen by HBcAg 140–155 peptide–specific lines and clones demonstrates that the CTL epitope represented by the core sequence 140–155 is actually generated by the intracellular processing of endogenously synthesized HBV core and precore proteins, and that these CTL are likely primed in vivo during HBV infection. The latter argument is confirmed by our repeated failure to establish HBcAg 140–155-specific CTL lines from six HLA-A31-positive or from four HLA-Aw68-positive normal uninfected controls that we have studied thus far (not shown).

The 11-mer HBcAg 141–151 Is the Minimum, Optimally Recognized HLA A31- and Aw68-restricted Epitope within HBcAg 140–155. Carboxy- and amino-terminal truncations of the core 140–155 sequence were produced to map the CTL epitopes that are restricted by HLA-A31 and Aw68 within this 16-residue peptide (Table 3). Clone 3D11, which is HLA A31 restricted, and clone 2D7, which is HLA Aw68 restricted, were used to define the fine specificity of the CTL response. Autologous B-LCL were preincubated with the truncated peptides (10 μM) and used as targets with the two clones. We

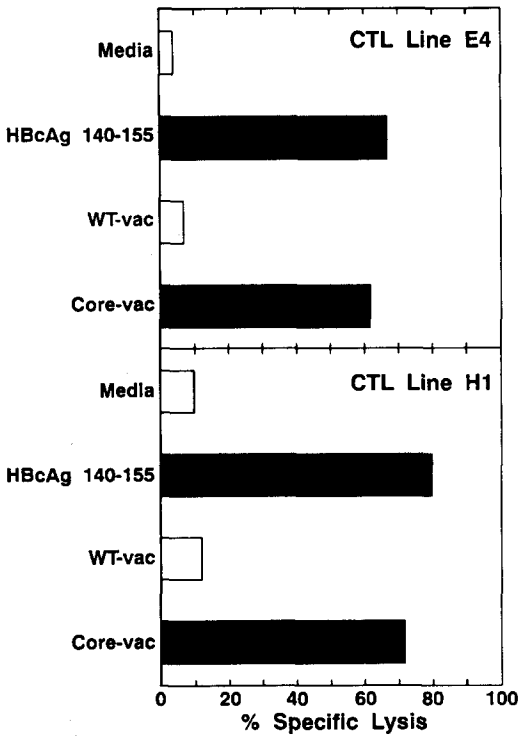

Figure 5. Recognition of endogenously synthesized HBcAg by HBcAg 140–155-specific CTL lines E4 (HLA-A31 and Aw68 restricted) and H1 (HLA-Aw68 restricted). CTL lines were incubated for 4 h with ^{51}Cr-labeled autologous B-LCL (line E4) and allogeneic B-LCL (line H1) matched at HLA-Aw68, which had been prepulsed with media or HBcAg 140–155 peptide, or were infected with recombinant vaccinia viruses that express the HBV core protein. The E/T ratio used for line E4 was 10:1, for line H1 was 20:1 for the peptide prepulsed target cells, and was 15:1 for the recombinant vaccinia–infected target cells.

Table 2. *HBcAg 140–155-specific Cytotoxicity of CTL Lines from HLA-A31- and Aw68-positive Patients with Acute HBV Infection*

		Target	
Patient	HLA match	HBcAg 140–155	Media
V.T.	A31	75	34
H.F.	All	10	1
Q.M.	Aw68	23	0
C.N.	Aw68,A24	25	10

PBMC stimulated with HBcAg 140–155 plus rHBcAg. Data represent percent ^{51}Cr release.

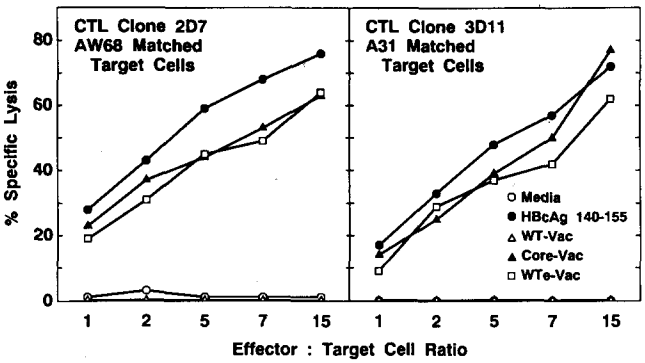

Figure 6. HLA-restricted recognition of endogenously synthesized HBcAg by CTL clones 2D7 and 3D11. Clones 2D7 and 3D11 were tested against HLA-Aw68- and A31-matched allogeneic target cells, respectively. Targets were either preincubated with peptide HBcAg 140–155, or infected with recombinant vaccinia viruses coding for the core (*Core-Vac*) or the precore (*WTe-Vac*) proteins of HBV in a 4-h ^{51}Cr release assay.

Table 3. *Fine Specificity of the Cytotoxic Activity of Clones 3D11 and 2D7*

Peptides		3D11	2D7
Residues	Sequence	A31	Aw68
140–155	LSTLPETTVVRRRGRS	72	62
140–154	LSTLPETTVVRRRGR	63	60
140–153	LSTLPETTVVRRRG	75	66
140–152	LSTLPETTVVRRR	77	69
140–151	LSTLPETTVVRR	72	67
140–150	LSTLPETTVVR	0	6
141–155	STLPETTVVRRRGRS	81	66
141–154	STLPETTVVRRRGR	79	67
141–153	STLPETTVVRRRG	79	59
141–152	STLPETTVVRRR	68	68
141–151	STLPETTVVRR	69	66
141–150	STLPETTVVR	20	52
141–149	STLPETTVV	0	3
142–155	TLPETTVVRRRGRS	8	63
142–154	TLPETTVVRRRGR	18	54
142–153	TLPETTVVRRRG	8	56
142–152	TLPETTVVRRR	2	37
142–151	TLPETTVVRR	47	60
142–150	TLPETTVVR	0	0
143–155	LPETTVVRRRGRS	0	0
143–154	LPETTVVRRRGR	0	0
143–153	LPETTVVRRRG	0	0
143–152	LPETTVVRRR	0	2
143–151	LPETTVVRR	0	0

Autologous target cells were preincubated with peptides at 10 μM; E/T ratio, 10:1. Lysis was of target cells preincubated with media <2% in all cases. Data represent percent ^{51}Cr release.

expected to identify two partially overlapping epitopes in peptide HBcAg 140–155. Surprisingly, however, our data indicate that sequence 141–151 is the minimal, optimally recognized epitope for both restriction elements. Initial studies using the truncated peptides at a single concentration (Table 3) defined residue 151 (arginine) as the putative carboxy terminus of the epitope recognized by both CTL clones. Interestingly, residue 150, which is also an arginine, can also serve as the carboxy-terminal residue for both clones but only if residue 141 (serine) serves as amino terminus. Although serine 141 appears to be the optimal amino-terminal residue of the epitope for both restriction elements, residue 142 (threonine) can also serve as the amino terminus of the epitope for both clones if arginine 151 is the carboxy-terminal residue. In contrast, only the HLA-Aw68-restricted clone (2D7) can use threonine 142 if the carboxy terminus of the peptide is extended beyond residue 151. The chemical similarity of the two amino-terminal residues (serine 141, threonine 142) and the identity of the two carboxy-terminal residues (arginine 150, 151) probably contribute to these observations.

To precisely define the boundaries of the epitope(s), a dose titration analysis was conducted in which the two CTL clones were incubated with allogeneic HLA-A31- and HLA-Aw68-positive target cells preincubated with peptides 140–155, 140–153, 140–151, 141–151, 141–150, 141–152, and 142–151 at different molar concentrations ranging from 10^{-3} to 10 μM. From these studies it is clear that core residues 141–151 represent the minimal optimally recognized epitope recognized by both of the CTL clones (Fig. 7), suggesting that both HLA alleles bind and present exactly the same peptide to their corresponding CTL. The availability of HLA-Aw68.1- and HLA-Aw68.2-positive cell lines permitted us to define the HLA-Aw68 subtype responsible for presentation of HBcAg 141–151 to clone 2D7. HLA Aw68.1-positive target cells pulsed with HBcAg 141–151 (1 μM) were efficiently lysed (39% specific ^{51}Cr release) by clone 2D7 at an E/T ratio of 10:1,

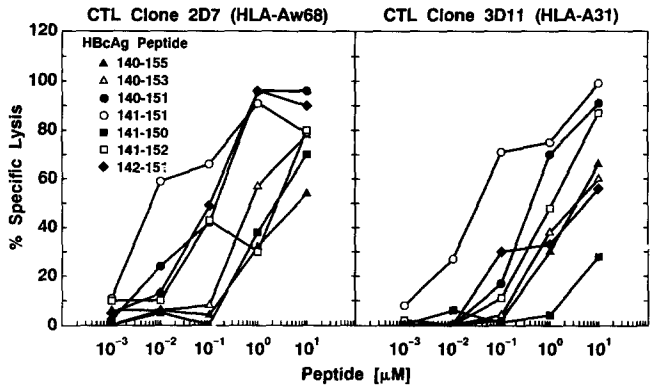

Figure 7. Identification of the shortest optimally recognized sequence in HBcAg 140–155 for each HLA restriction element. Clone 3D11 was tested against allogeneic ^{51}Cr-labeled A31-positive target cells and clone 2D7 against allogeneic ^{51}Cr-labeled Aw68-positive target cells prepulsed for 1 h with various peptides at concentrations ranging between 0.001 and 10 μM. Target cells were incubated in presence of peptides at 37°C for 1 h and subsequently diluted 20-fold in RPMI 1640 containing 10% FCS and added to the effector cells for 4 h at an E/T ratio of 10:1.

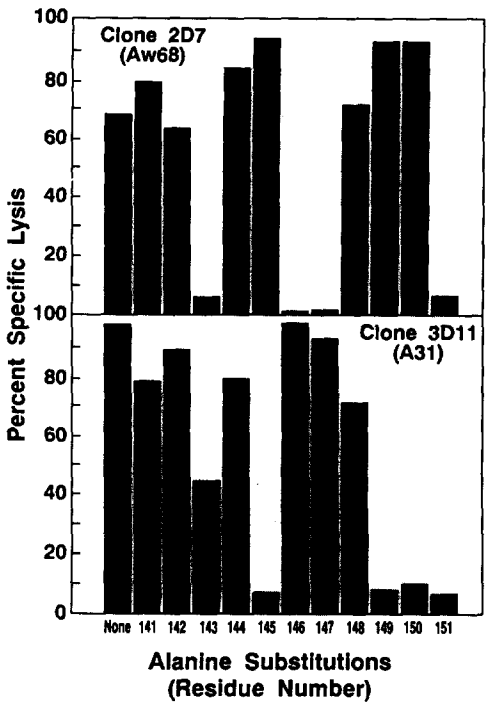

Figure 8. Recognition of HBcAg 141–151 peptides containing alanine substitutions at each residue. Identification of critical residues involved in presentation and recognition of HBcAg 141–151. Clone 3D11 was tested against ^{51}Cr-labeled allogeneic A31-positive target cells, and clone 2D7 against allogeneic ^{51}Cr-labeled Aw68-positive target cells that had been prepulsed for 1 h with varying concentrations (0.001–10 μM) of HBcAg 141–151 or with a panel of modified peptides in which alanine was substituted at each position. Target cells were incubated in presence of peptides at 37°C for 1 h and subsequently diluted 20-fold in RPMI 1640 containing 10% FCS and added to the effector cells for 4 h at an E/T ratio of 8:1. Representative results obtained at 1 μM peptide concentration are shown.

in contrast to HLA-Aw68.2-positive targets, which displayed no specific lysis under the same conditions (not shown).

Identification of Critical Residues Involved in Presentation and Recognition of HBcAg 141–151. To define the residues within HBcAg 141–151 that interact with the HLA-A31 and Aw68 molecules and that are recognized by the CTL receptors of clones 3D11 and 2D7, respectively, a series of peptides containing individual alanine substitutions at each residue (A141–A151) were produced. Allogeneic HLA-A31- and HLA-Aw68-matched target cells were pulsed with each peptide at multiple concentrations between 0.001 and 10 μM and used in a 4-h ^{51}Cr release assay with clones 3D11 and 2D7 at an E:T ratio of 10:1. Comparison of the dose-response curves for each substituted peptide revealed a hierarchy that is illustrated in Fig. 8, with the results obtained at a single peptide concentration (1 μM) for clarity. As shown in Fig. 8, the two clones displayed similar, but not identical, peptide recognition profiles. Interestingly, substitutions of leucine 143 and arginine 151 either reduced or nearly abolished CTL-mediated killing by both clones. In contrast to these shared substitutions, threonine 146 and threonine 147 substitutions almost completely abolished CTL-mediated killing by clone 2D7, but they had no effect on clone 3D11. On the contrary, substitution of glutamic acid 145, valine 149, and arginine 150 almost completely abolished recognition by 3D11 but had no effect on 2D7. Although it is clear that these residues are involved in peptide interaction with either the HLA class I restriction elements or with the TCRs of the corresponding CTL clones, the fact that leucine 143 and arginine 151 appear to be important for both HLA-A31- and the HLA-Aw68-restricted responses suggests to us that these residues may mediate the binding of HBcAg 141–151 to both HLA alleles (agretope), while threonine 146 and 147 may represent residues that interact with the TCR of clone 2D7 (epitope), whereas glutamic acid 145, valine 149, and arginine 150 might contribute to the epitope recognized by the 3D11 TCR.

To distinguish between HBcAg 141–151 residues that interact with the HLA-A31 and Aw68 molecules and those residues that are seen by the CTL receptor in clones 3D11 and 2D7, we examined the ability of alanine-substituted peptides (100 μM) to prevent the binding of the native HBcAg 141–151 peptide (0.05 and 0.01 μM, respectively) to HLA-A31- and HLA-Aw68-positive target cells (Fig. 9).

Our results demonstrate that alanine substitutions of glutamic acid 145 and valine 149 effectively block CTL recognition of HBcAg 141–151 by HLA-A31-restricted clone 3D11, indicating that they probably represent TCR binding residues (epitope). In contrast, peptides containing alanine substitutions at residues leucine 143 and arginine 150 and 151 do not block the binding of HBcAg 141–151 to the HLA-A31 molecule, suggesting that some or all of these three residues are probably involved in the interaction of HBcAg 141–151 with HLA-A31. The results are particularly convincing for positions 150 and 151 because the corresponding alanine-substituted peptides do not sensitize the target cells to lysis by clone 3D11 at the high concentrations used for competition in this experiment. However, because the alanine 143–substituted peptide itself was recognized at the high con-

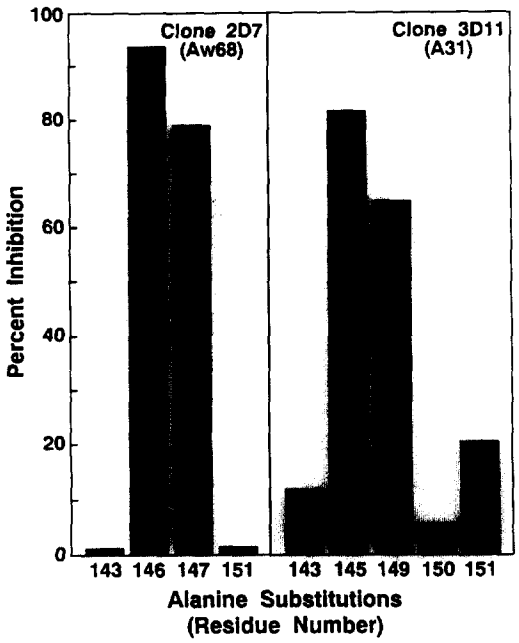

Figure 9. Competitive inhibition binding of HBcAg 141–151 to HLA-Aw68 and A31. Identification of residues within HBcAg 141–151 responsible for interaction with HLA-A31 and HLA-Aw68, and with the CTL receptors in clones 2D7 and 3D11. Peptides containing alanine substitutions at the indicated positions (100 μM) were added to HLA-Aw68- or HLA-A31-positive target cells for 10 min, after which HBcAg 141–151 was added as a target peptide at 0.01 or 0.05 μM, respectively. 1 h later the target cells were diluted 20-fold in complete media and the corresponding CTL clones were added at an E/T ratio of 10:1 in a 4-h ^{51}Cr release assay. Results are expressed as percent inhibition of the HBcAg 141–151-specific lysis observed in the absence of competitor peptide.

centrations required for this experiment, additional experiments were performed using a peptide with a nonconservative substitution (glutamic acid) at position 143. This peptide did not sensitize target cells even at high concentrations and it did not compete with the unsubstituted HBcAg 141–151 when it was used at 100-fold molar excess. Thus, the data suggest that leucine 143 and arginine 150 and 151 are probably HLA-A31 contact residues.

For the HLA-Aw68-restricted CTL clone 2D7, alanine substitution of threonine 146 and threonine 147 effectively block CTL recognition of HBcAg 141–151, indicating that these residues probably represent a TCR residue (epitope). On the other hand, peptides containing alanine substitutions at residues leucine 143 and arginine 151 do not block the binding of HBcAg 141–151 to the HLA-Aw68 molecule, even at 10,000-fold molar excess (concentrations that are entirely nonsensitizing for lysis by clone 2D7). These data strongly suggest that both of these residues are necessary for interaction of HBcAg 141–151 with HLA-Aw68.

Discussion

In the present study we have identified a new CTL epitope in the nucleocapsid protein of the HBV, corresponding to HBcAg residues 141–151, by synthetic peptide stimulation of PBL from patients with acute viral hepatitis. We have shown that this epitope is generated by the cellular processing of endogenously synthesized HBV core and precore proteins, and that the CTL response to this single 11-residue minimal epitope is restricted by two different HLA molecules, HLA-A31 and HLA-Aw68. Finally, we have defined, at least partially, the agretopic and epitopic residues in HBcAg 141–151 that are seen by two independently restricted CTL clones, and by so doing we have tentatively identified a common, allele-specific binding motif for HLA-A31 and Aw68.

As we have reported previously (12–14, 17), we used synthetic peptides to expand the quantitatively limited, peripheral blood CD8-positive HBV antigen-specific cytotoxic T cell population in our patients. This strategy is necessary because HBV does not readily infect human cell lines in vitro and because our panel of stable HBV transfectants (17) apparently do not express sufficient amounts of endogenously processed antigen on the cell surface for efficient in vitro secondary induction of an HBV antigen-specific, class I–restricted peripheral blood CTL response in the HBV-infected patients we have studied thus far. In contrast, CTL stimulation with synthetic peptides is a powerful strategy to expand antigen-specific CTL populations that have been primed in vivo since we have shown, here and elsewhere (14, 17), that the peptide-stimulated CTL can efficiently recognize targets that express endogenous antigen. It is interesting that the stable transfectants can be very effectively used to select, expand, and maintain CTL that preferentially recognize endogenously synthesized antigen from the peptide-stimulated CTL population despite their inability to expand the same antigen-specific CTL without prior stimulation by the synthetic peptides. This may reflect the requirement for a higher epitope density for induction of a CTL response than for its maintenance, as previously described (14, 17). Nonetheless, the use of these transfectants for this purpose is important because it selects for the expansion of CTL populations that recognize endogenously synthesized epitopes rather than those subpopulations of CTL that only recognize the peptide used for the initial expansion.

It is noteworthy that the current stimulation strategy has permitted us to detect a CTL response to two independent epitopes in patient H.P., who displayed a response to the HLA-A2-restricted HBcAg 18–27 epitope that we have previously reported (12–14, 17) and to the HLA-Aw68-restricted epitope HBcAg 141–151 reported herein. Furthermore, patient E.W. yielded two independent CTL clones whose antigenic fine specificity differed according to the HLA allele that restricted the response (glutamic acid 145 and valine 149 for the HLA-A31-restricted clone, and the two threonine residues at positions 146 and 147 for the clone restricted by HLA-Aw68). These data, and others not presented in this study (F. V. Chisari et al., unpublished observations), suggest that the CTL response to HBV in humans is both polyvalent and multispecific, presumably to afford efficient protection from this serious viral infection.

Another interesting result is the efficient CTL recognition of target cells expressing endogenously synthesized core

and precore proteins (Fig. 6), which are identical except for the presence of a 29-residue amino-terminal signal sequence in the precore protein that directs it to the endoplasmic reticulum where 19 amino-terminal residues and 34 carboxy-terminal residues (residues 149–183) are removed and the product is secreted via the Golgi apparatus into the serum as HBeAg. In contrast, the core protein lacks the signal sequence and is targeted principally to the nucleus by a series of arginine-rich localization signals at its carboxy terminus (20); one of which (residues 145–156) also plays a critical role in viral RNA encapsidation (21). It is interesting that residues 141–151 are involved in all of these important events in the virus life cycle. The significance of this observation is not known at present. However, the CTL response to this epitope could be especially effective at viral clearance because in addition to destroying HBV-infected cells, CTL-resistant "escape" mutants at this epitope would probably be nonviable. The current data indicate that, despite their different intracellular trafficking pathways, the core and precore proteins appear to intersect either at the level of proteolytic processing in the cytoplasm or at the level of MHC binding within the endoplasmic reticulum since target cells that synthesize either protein are equally recognized by HBcAg 18–27 (14) and HBcAg 141–151-specific CTL (Fig. 6).

Relatively few antigenic determinants have been described that are broadly presented by multiple MHC molecules. In most instances the responses have been class II restricted (22, 23), although a murine CTL epitope in HIV-1 envelope glycoprotein has been recently described that can be presented by at least four different class I MHC molecules from independent haplotypes (24). However, at the human class I–restricted CTL level, broadly restricted responses have been described only for partially overlapping epitopes (25), or for antigens in which the minimal CTL epitope has not been precisely determined (26). To our knowledge, therefore, this is the first report that a single, precisely defined CTL epitope generated by the intracellular processing of an endogenously synthesized protein effectively induces a specific CTL response that is restricted by two independent HLA class I alleles in vivo in humans.

Insight into the molecular basis for this event can be gained by comparing the structural and chemical properties of the antigen-binding pockets in the HLA molecule with the results of our alanine substitution experiments. The polymorphic HLA class I protein is characterized by 20 highly variable residues, 16 of which are thought to contribute to formation of at least two pockets that have been identified in the antigen binding groove of the HLA-A2 and HLA-Aw68 molecules (27–30). These pockets are presumed to represent the binding sites of critical anchor residues within antigen peptides that mediate their binding to the HLA class I molecule.

Structural analysis reveals that one of these pockets is formed by hydrophobic residues at positions 24, 26, 34, and 67 of the class I heavy chain plus a highly variable residue at position 45 (30). Comparative DNA sequence analysis reveals that this pocket should be present in all HLA-A molecules, except perhaps HLA-A1 (31). The existence of a hydrophobic pocket in the HLA-A2 protein is compatible with recent observations that leucine and other hydrophobic amino acids located at position 2 represent a critical anchor residue for antigenic nonamer and decamer peptides that can be eluted from the HLA-A2.1 binding groove (32, 33). It is also consistent with our previous identification of leucine at position 2 in a 10–amino acid HBcAg-derived sequence (HBcAg 18–27) (FLPSDFFPSV) that is a major, HLA-A2-restricted CTL epitopes in patients with acute viral hepatitis (12).

It is important to note that leucine 143 is present as residue 3 of the HLA-A31- and HLA-Aw68-restricted CTL epitope described in the current report (STLPETTVVRR). This observation raises the possibility that leucine 143 may contribute, as an anchor residue, to the binding of this peptide to both restriction elements. Indeed, our experimental results (Figs. 8 and 9) suggest that this is true although the relative importance of this residue in binding to the HLA-A31 and Aw68 molecules may be somewhat different.

Structural analysis of the HLA-Aw68 protein reveals the presence of a prominent negatively charged pocket, defined by an aspartic acid in position 74 in both HLA-Aw68.1 and 68.2 subtypes, plus two residues at positions 97 and 116, which differ between the two subtypes (Aw-68.1, methionine and aspartic; Aw68.2, arginine and tyrosine). Because of these differences, positively charged residues like arginine, in antigenic peptides, have access to the 74 pocket in HLA-Aw68.1 but not in Aw68.2 (31). Comparative DNA sequence analysis reveals that residues 74, 97, and 116 are all conserved in HLA-Aw68, A29, A31, A32, and A33 molecules but not in other class I alleles.

Since a positively charged carboxy-terminal residue (arginine 151) is required for recognition of the current epitope by both HLA-Aw68- and A31-restricted CTL (Table 3 and Figs. 7 and 8), it is possible that it represents a second important anchor residue required for peptide interaction with both of these HLA molecules. This is supported by the alanine substitution studies, which demonstrate that arginine 151 is critical for presentation of HBcAg 141–151 to both CTL clones (Fig. 8) but that it does not behave as an epitopic residue in blocking studies illustrated in Fig. 9, suggesting that it is probably involved in interaction with the HLA class I molecules. The data also suggest that arginine 150 may bind to HLA-A31 but not to Aw68 (Figs. 8 and 9). It is pertinent to point out, at this point, that an arginine has also been suggested to be the anchor residue for the 74 pocket of HLA-Aw68 for an influenza CTL epitope (19). Furthermore, the inability of HLA-Aw68.2-positive target cells to present HBcAg 141–151 to clone 2D7, while HLA-Aw68.1-positive target cells are fully functional, supports our belief that the carboxy-terminal arginine anchors HBcAg 141–151 to the 74 pocket of the HLA-Aw68.1 molecule.

As mentioned above, our data suggest that the epitopic residues within HBcAg 141–151 that interact with the CTL receptor are different for the two clones we have studied. The HLA-A31-restricted CTL clone 3D11 recognizes glutamic acid 145 and valine 149 while the HLA-Aw68-restricted CTL clone 2D7 recognizes the two threonine residues at positions 146

and 147. It will be interesting to generate additional HLA-A31- and Aw68-restricted, HBcAg-specific CTL clones in the future to assess the degree to which the CTL repertoire varies with respect to recognition of this HBV-encoded antigen.

Finally, the identification of this new CTL epitope in the HBV nucleocapsid protein is relevant to HBV immunobiology and pathogenesis for several reasons.

First, it expands our knowledge of the HBV-specific CTL repertoire to a new domain within the viral nucleocapsid protein and to two new HLA class I restriction elements.

Second, it demonstrates, for the first time, that the HBV-specific CTL response during acute viral hepatitis extends to multiple viral epitopes and different restriction elements; i.e., it is polyclonal, multispecific, and polymorphically restricted, thereby affording enhanced protection for the host against a pathogen that is as dangerous and as prone to mutate as HBV.

Third, it demonstrates that the HBV core and precore proteins are equally good targets of a nucleocapsid-specific CTL response to a shared epitope such as HBcAg 141–151, as we have previously demonstrated for HBcAg 18–27 (14). This implies that viruses containing mutations that affect only one of these proteins, such as the well-described precore stop codon mutation that blocks precore protein translation without affecting the core protein (34, 35), probably do not represent CTL escape mutants, since cells infected by the mutant viruses should still produce the HBcAg 141–151 epitope and be eliminated by the CTL. This further implies that factors other than CTL-imposed selection pressure are probably responsible for emergence of the precore codon 28 mutants that appear to be more virulent than wild-type forms of HBV (35).

Fourth, it focuses our attention on a functionally important domain within the HBV nucleocapsid region that is involved not only in nuclear localization of HBcAg (20) but also in viral RNA encapsidation (21), and also in cleavage of the precore protein to produce HBeAg (36). Empirically, generation of a CTL response to this particular domain would afford a double benefit to the host, first, by destroying the infected cells and, second, by selecting for nonviable mutants since at least two of these functions are known to be important in the viral life cycle.

Finally, if we can demonstrate that chronically HBV-infected HLA-A31- and Aw68-positive patients respond poorly to HBcAg 141–151, as we have demonstrated for the HLA-A2-restricted response to HBcAg 18–27 (13), it may represent a second potential target for future CTL-based, immunotherapeutic strategies designed to terminate chronic HBV infection. Since such strategies are HLA haplotype dependent, the known world-wide prevalence of the HLA-A2, HLA-A31, and Aw68 alleles (37) suggests that the successful induction of a CTL response to HBcAg 18–27 could benefit ~45% of chronically infected patients, and that another 15–20% could benefit from induction of a therapeutic HBcAg 141–151-specific CTL response.

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Note added in proof: While this manuscript was under review, Guo et al. and Silver et al. (38, 39) demonstrated, by structural analysis, that the HLA-Aw68.1 molecule binds peptides containing a threonine residue in position 2, a hydrophobic residue (such as leucine) in position 3, and an arginine residue at the carboxy terminus. Since HBcAg 141–151 contains this precise motif, and since we had not identified threonine 142 as an important binding residue for HLA-Aw68, or for HLA-A31, in our alanine substitution studies (Fig. 8), we examined the ability of a peptide containing a nonconservative tryptophan residue at this position (T142W) to prime target cells for recognition by clones 2D7 (HLA-Aw68 restricted) and 3D11 (HLA-A31) restricted). In a peptide dose titration experiment we recently determined that peptide T142W was recognized 100–1,000-fold less efficiently by clones 2D7 and 3D11 in comparison with the native peptide (not shown). These new findings are compatible with the recently published structural data mentioned above (38, 39), and they suggest that threonine in position 2 may also serve as an anchor residue for the binding of immunogenic peptides to HLA-A31 as well.

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