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HLA A2 Restricted Cytotoxic T Lymphocyte Responses to Multiple Hepatitis B Surface Antigen Epitopes during Hepatitis B Virus Infection[1]

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Abstract. Inasmuch as the hepatitis B virus (HBV) is not directly cytopathic for the infected hepatocyte, it is generally presumed that viral clearance and liver cell injury during viral hepatitis are due to a CTL response to HBV encoded Ag presented by HLA class I molecules. We have previously examined the peripheral blood CTL response to two HBV nucleocapsid epitopes in patients with acute and chronic viral hepatitis, one of which is restricted by HLA-A2, whereas the other is dually restricted by HLA-A31 and Aw68. In this study, we defined the HLA-A2-restricted CTL response to the hepatitis B surface Ag (HBsAg) by using a panel of HBsAg-derived synthetic peptides containing the ideal HLA-A2.1 binding motif (-L - - - - -V). Several novel aspects of HBV immunobiology and pathogenesis are evident from this study. First, the peripheral blood CTL response to HBV-encoded Ag is remarkably polyclonal and multispecific in most patients with acute hepatitis. Indeed, HLA-A2-restricted CTL specific for as many as four envelope epitopes and one nucleocapsid epitope were found to be present simultaneously in individual patients with acute viral hepatitis. Second, HBV-specific CTL are not detectable in the peripheral blood in a minority of patients with acute hepatitis, nor have we detected a CTL response in any of the patients with chronic hepatitis that we have studied thus far. Although the cellular and molecular basis for CTL nonresponse remains to be determined, the data suggest that it may contribute to viral persistence. Third, the diversity and the specificity of the CTL response is determined in part by the coding sequence of the viral genome present in each infected patient. Indeed, the apparent nonresponse of some acutely infected patients to at least one HBsAg-specific CTL epitope actually reflects infection by a viral variant that contains a critical substitution in one of the anchor residues within the epitope. Finally, at a fundamental level, the data suggest that the presence of the HLA-A2.1-binding motif in a peptide may not be sufficient for binding; and the capacity of a peptide to bind the class I molecule does not guarantee that it will be immunogenic. *Journal of Immunology,* 1993, 150: 4659.

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The HBV[4] is a small, enveloped virus with a double-stranded DNA genome (1), which causes acute and chronic hepatitis and hepatocellular carcinoma (2). Because it is generally acknowledged that HBV is not directly cytopathic for the infected hepatocyte (2), the cellular immune response to HBV-encoded Ag may be responsible for HBV-induced liver disease and it probably plays an important role in viral clearance as well.

Because HLA class I restricted CTL are known to interact with processed viral peptides bound to class I molecules at the surface of infected cells (3, 4), it is generally assumed that they play a direct role in viral clearance and tissue injury by destruction of infected cells (5) and possibly by non-cytolytic anti-viral mechanisms as well (6, 7). Accordingly, we have begun to examine the characteristics and consequences of the $CD8^+$, HLA class I-restricted, HBV-specific CTL response in infected patients (8–12) and in HBV transgenic mice (13).

By using a strategy involving in vitro stimulation of PBMC with HBV-derived synthetic peptides, we have reported that more than 90% of HLA-$A2^+$ patients with acute HBV infection produce an HLA-A2-restricted, $CD8^+$ CTL response to a 17-residue peptide corresponding to amino acids 11–27 of HBcAg, whereas chronically infected patients do not respond to this epitope (11). Recently, we demonstrated that the $HBcAg_{11-27}$-specific response is focused on a 10- residue epitope that maps to amino acids 18–27 (FLPSDFFPSV) of HBcAg (8). Importantly, this 10 mer almost perfectly reiterates the ideal HLA-A2.1 allele-specific binding motif which has been described as a 9 mer-containing leucine and valine as dominant anchor residues at these positions by Rammensee's group (14).

HBV encodes three envelope proteins that share a common 226-amino acid carboxy terminus containing the HBsAg. Because the envelope proteins are expressed at high levels by infected hepatocytes, and because HBsAg is especially rich in hydrophobic leucine and valine residues, we have used the same peptide stimulation strategy to search for HLA-A2-restricted, envelope-specific CTL in the peripheral blood of HLA-$A2^+$ patients with acute and chronic hepatitis.

In this paper we report, for the first time, that most patients with acute viral hepatitis produce a CTL response to several HLA-A2-restricted epitopes within HBsAg, and that patients with chronic hepatitis do not. We demonstrate that the CTL response is both polyclonal and multispecific within individual patients, and we show that the HBsAg-derived epitopes are generated by the cellular processing of all three envelope polypeptides. Collectively, these data suggest that viral escape mutants are not likely to have a selective survival advantage in this setting. We also demonstrate that both the generation and the epitope specificity of an HBsAg-specific CTL response in individual patients is determined by the coding sequence of the virions with which they are infected and that it is influenced by the HLA-A2-binding affinity of the derivative HBsAg peptides and by host factors, presumably at the level of Ag processing and the T cell repertoire.

[4] Abbreviations used in this paper: HBV, hepatitis B virus; HBsAg, hepatitis B surface Ag; HBcAg, hepatitis B core Ag; rHBcAg, recombinant hepatitis B core Ag; B-LCL, B lymphoblastoid cell line; ALT, alanine aminotransferase; PCR polymerase chain reaction.

Table I
Characteristics of subjects studied

Subject	Sex	Diagnosis	HLA Class I Haplotype
A-1	Male	Acute	A2, A30, B35, B44, Cw4, Cw7
A-2	Male	Acute	A2, A31, 8w58(5Y), B51, Cw3
A-3	Male	Acute	A2, Bw41, Bw71, Cw4, Cw7
A-4	Male	Acute	A2, A32, Bw41, Bw71, Cw4, Cw7
A-5	Male	Acute	A2, A1, B8, 8w58(5Y), Cw7
A-6	Female	Acute	A2, Aw68, B35, Cw3, Cw4
A-7	Male	Acute	A2, A1, B8, Bw73, Cw3, Cw4
A-8	Female	Acute	A2, Aw69, Bw53, Cw4
A-9	Male	Acute	A2, A24, B7, B27, Cw2, Cw7
A-10	Male	Acute	A2, A3, Bw62, Bw71, Cw3, Cw4
A-11	Male	Acute	A2, A24, B35, Cw4
A-12	Male	Acute	A2, A3, Cw5
A-13	Male	Acute	A2, A3, B7, Bw60, Cw3, Cw7
C-1	Male	Chronic	A2, B27, B35, Cw2, Cw4
C-2	Male	Chronic	A2, A1, B8, B44
C-3	Male	Chronic	A2, A24, B44, Bw67
C-4	Male	Chronic	A2, Aw69, Bw41, Bw52
C-5	Male	Chronic	A2, B5, Bw62, Cw4
C-6	Male	Chronic	A2, A26, B35, Cw4
N-1	Male	Normal	A2, A11, B44, Cw4
N-2	Male	Normal	A2, Bw56, B35
N-3	Male	Normal	A2, A11, B8, Bw62, Cw4
N-4	Male	Normal	A2, A23, B5, Bw58, Cw2, Cw6
N-5	Male	Normal	A2, B44, Bw63
N-6	Female	Normal	A2, A11, Bw58

Materials and Methods

Patient population

All patients included in this study were HLA-$A2^+$. Thirteen patients (A-1 to A-13) were studied during an episode of acute hepatitis B, six patients (C-1 to C-6) were chronically infected by HBV, and six uninfected healthy volunteers (N-1 to N-6) served as normal controls (Table I).

The diagnosis of acute hepatitis B was based on standard diagnostic criteria as we have previously described (10). Diagnostic parameters included clinical (jaundice) and biochemical evidence of liver cell injury (ALT activity at least 20-fold greater than the upper limits of normal), together with serologic evidence of acute HBV infection (presence of HBsAg and IgM anti-HBc antibody) in the absence of serologic evidence of hepatitis δ and hepatitis C virus infection (Abbott Laboratories, North Chicago, IL). All patients were studied during the first 4 wk after the onset of jaundice, at which time their serum was positive for HBsAg and their ALT levels were markedly abnormal. Eleven of

Table II
HBV-derived ideal HLA-A2.1-binding motifs used in this study

	Peptide	Sequence
1	$HBcAg_{18-27}$	FLPSDFFPSV
2	$HBsAg_{201-210}$	SLNFLGGTTV
3	$HBsAg_{251-259}$	LLCLIFLLV
4	$HBsAg_{260-269}$	LLDYQGMLPV
5	$HBsAg_{335-343}$	WLSLLVPFV
6	$HBsAg_{338-347}$	LLVPFVQWFV
7	$HBsAg_{348-357}$	GLSPTVWLSV
8	$HBsAg_{378-387}$	LLPIFFCLWV

the 13 patients subsequently recovered completely from the illness, with normalization of serum transaminase and clearance of HBsAg within 4 mo of initial diagnosis. One patient (A-11, Table I) developed chronic active hepatitis and remained $HBsAg^+$ 13 mo after initial diagnosis. One patient (A-10, Table I) was lost to follow-up after his initial clinic visit. Patients with chronic hepatitis B were repeatedly serologically positive for HBsAg for more than 6 mo and displayed mildly to moderately elevated serum ALT activity. Normal controls had no clinical history of HBV infection and they were serologically negative for HBV markers. All patients and normal controls were serologically negative for antibody to HIV.

Synthetic peptides and HBV Ag

In this report we will define any 9- or 10-residue peptide that contains a leucine in the second position and a valine at the carboxy terminus as an "ideal HLA-A2.1-binding motif." A panel of synthetic peptides containing the ideal HLA-A2.1-binding motif (Table II) was provided by Cytel Corporation (San Diego, CA) or purchased from Multiple Peptide Systems (San Diego, CA) or Chiron Mimotopes (Clayton, Australia). Lyophilized peptides were reconstituted at 20 mg/ml in DMSO (Malinckrodt, Paris, KY) and diluted to 1 mg/ml with RPMI 1640 medium (GIBCO, Grand Island, NY). rHBcAg was obtained from bacterial extracts of *Escherichia coli* as previously described (15).

Stimulation of PBMC with synthetic peptides and rHBcAg

PBMC from patients and normal donors were separated on Ficoll-Hypaque density gradients (Sigma, St. Louis, MO), washed three times in HBSS (GIBCO), resuspended in RPMI 1640 medium (GIBCO) supplemented with L-glutamine (2 mM), gentamicin (10 μg/ml), penicillin (50 U/ml), streptomycin (50 μg/ml), and HEPES (5 mM) containing 10% heat-inactivated human AB serum (complete medium) and plated in 24-well plates at 4×10^6 cells/well. The synthetic peptides were added to the cell cultures at a final concentration of 10 μg/ml unless otherwise noted. rHBcAg was added at 1 μg/ml during the first week of stimulation as we have previously described (10). At day 3, 1 ml of complete medium supplemented with rIL-2 (Hoffmann-La Roche, Nutley, NY) at 10 U/ml final concentration was added in each well. On day 7, the cultures were restimulated with peptide, rIL-2, and irradiated (3,000 rad) autologous or HLA-A2-matched feeder cells, and the cultured PBMC were tested for CTL activity on day 14. Selected cultures that displayed peptide specific cytolytic activity were expanded by weekly restimulation with 1×10^6 irradiated (6,000 rad) allogeneic PBMC and 1×10^5 irradiated (18,000 rad) JY cells (allogeneic EBV-B-transformed cell line, HLA-A2.1, B7, Cw7) (16) in 1 ml of complete medium containing 1 μg/ml peptide, 20 U/ml IL-2, and 1 μg/ml PHA (Sigma).

Generation of HBV-specific CTL clones

CTL lines were cloned originally at 1, 10, and 100 cells/well and then subcloned at 0.3 or 1 cell/well in 96-well microtiter plates. The cells were plated in the presence of peptide (1 μg/ml), PHA (1 μg/ml), rIL-2 (20 U/ml), irradiated (6,000 rad) allogeneic PBMC (10^5 cells/well), and irradiated (18,000 rad) JY cells (10^4 cells/well). HBV-specific clones were restimulated in a 24-well plate as described above except that the peptide was omitted and irradiated JY cells, transfected with a plasmid that confers stable expression of the HBV large envelope Ag (EBO-preS1, Ref. 12), were added at 10^5 cells/well.

Target cells

Autologous and allogeneic EBV-transformed B-LCL were either purchased from The American Society for Histocompatibility and Immunogenetics (Boston, MA) or established from our own pool of patients and normal donors as previously described (17). The cells were maintained in RPMI 1640 supplemented with L-glutamine (2 mM), gentamicin (10 μg/ml), penicillin (50 U/ml), streptomycin (50 μg/ml), HEPES (5 mM), and 10% (vol/vol) heat-inactivated FCS (GIBCO). Short term lines of autologous PBMC blasts were produced by stimulating PBMC with PHA at 1 μg/ml in the RPMI 1640 supplemented with L-glutamine (2 mM), gentamicin (10 μg/ml), penicillin (50 U/ml), streptomycin (50 μg/ml), HEPES (5 mM), 10% (vol/vol) heat inactivated FCS, and 10 U/ml rIL-2 for 7 days before use as target cells (see below).

Recombinant expression vectors

Recombinant vaccinia viruses expressing the HBV large, middle, and major envelope polypeptides (adw subtype) and a corresponding control wild-type vaccinia were generously provided by Dr. Bernard Moss (18–20). An independent series of recombinant vaccinia viruses expressing the same three HBV envelope polypeptides of the HBV ayw subtype was derived as follows. For expression of the

HBsAg, an *Xho*I/*Sph*I restriction fragment containing nucleotides 1409 to 2514 of the HBV sequence was cloned into a vaccinia virus expression vector downstream from the 7500 early/late promoter. For the preS1 expressing vaccinia virus, a *Bgl*II/*Sph*I fragment containing nucleotides 937 to 2514 was used. For cloning of the preS2 coding sequence, first a short adapter oligonucleotide was synthesized which started at nucleotide 1267 (e.g., 6 bp upstream from the preS2 start codon) and spanned the *Eco*RI site at position 1280. After cloning of this oligonucleotide into the vaccinia virus expression vector, the coding sequence was completed by recloning of the *Eco*RI/*Sph*I HBV fragment (nucleotides 1280–2514) into this intermediate construct. Generation of recombinant vaccinia viruses was done according to standard procedures as previously described (18). Stable transfectants that express the HBV envelope proteins (ayw subtype) were produced by transfection of EBV-transformed B-LCL with a panel of EBV-based expression vectors that contain the corresponding HBV (ayw subtype) coding regions as previously described (12).

Cytotoxicity assay

Target cells consisted either of 1) autologous PHA-stimulated blasts or allogeneic HLA-matched and mismatched B-LCL incubated overnight with synthetic peptides at 10 μg/ml; 2) stable B-LCL transfectants described above; or 3) B-LCL infected with recombinant vaccinia viruses. Vaccinia-infected targets were prepared by infection of 1×10^6 cells at 50 plaque-forming U/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 labeled with 100 μCi of ^{51}Cr (Amersham, 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 by using U-bottom 96-well plates containing 5000 targets/well. All assays were performed in duplicate. Percent cytotoxicity was determined from the formula:

$$100 \times \left[\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}\right].$$

Maximum release was determined by lysis of targets by detergent (1% Triton X-100; Sigma). Spontaneous release was less than 25% of maximal release in all assays.

Competitive binding inhibition assay

The relative HLA-A2.1-binding affinity of selected peptides was measured by assessing their ability to competitively inhibit the binding of a known HLA-A2-restricted CTL epitope ($HBcAg_{18-27}$) to a homozygous HLA-A2.1$^+$ EBV-transformed target cell line (JY). An $HBcAg_{18-27}$-specific CTL clone from patient A-4 was used as a source of effector cells for these experiments. Blocking peptides (1, 10, 100 μM) were added to a mixture of ^{51}Cr-labeled, HLA-A2.1$^+$ JY target cells and effector cells for 40 min before the addition of a subsaturating concentration (0.03 μM) of the target peptide, $HBcAg_{18-27}$. The binding ability of each peptide was assessed by calculating the degree to which it blocked the lysis of target cells in a 4-h ^{51}Cr-release assay.

Flow cytometry analysis

Lines and clones were washed once in PBS and then incubated with a fluorescent probe-conjugated anti-CD4 (Leu-3a) and anti-CD8 (Leu-2a) mAb and similarly labeled control antibody (Becton Dickinson, Mountain View, CA). After a 1-h incubation at 4°C, in PBS with 5% BSA and 0.02% sodium azide, cells were washed three times and analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

HLA typing

HLA typing of PBMC from patients and from normal donors was determined by microcytotoxicity, with the use of HLA-typing trays (One Lambda, Canoga Park, CA). The HLA haplotypes of all subjects used in this study are shown in Table I.

DNA sequence analysis

The DNA sequence of HBV envelope codons 335 to 357 in virion DNA was derived from the serum of 10 of the acutely infected patients, and representative recombinant vaccinia viruses that express the HBV major envelope polypeptide of both the ayw and adw subtypes were derived by PCR amplification of the corresponding region of the viral genome followed by sequencing. All samples were digested with 20 μg proteinase K for 1 h at 45°C. After addition of 1 μg of *E. coli* tRNA as carrier, samples were phenol-extracted and ethanol-precipitated. Two 20-mer oligonucleotides (TCGGAAAATTCCTATGGGAG and TTAGGGTTTAAATGTATACC) representing nucleotides 625 to 644 and 840 to 821 of the HBV ayw sequence (21), respectively, were used to amplify a 215-nucleotide region of the HBV genome spanning the HBV envelope codons 335 to 357 by the PCR reaction (22). The amplification products were purified by electrophoresis into a 2% agarose gel and recovered onto glass beads by using an Elu-quik DNA purification kit (Schleicher and Schuell, Keene, NH) according to the manufacturer's recommendations. The purified fragments were sequenced by the chain termination method (23) with modified T7 DNA polymerase (Sequenase sequencing kit, U.S. Biochemical, Cleveland, OH) with the use of an internal oligonucleotide (GGGACTCAAGATGCTTGTACA), located between nucleotides 785 and 766, as a primer.

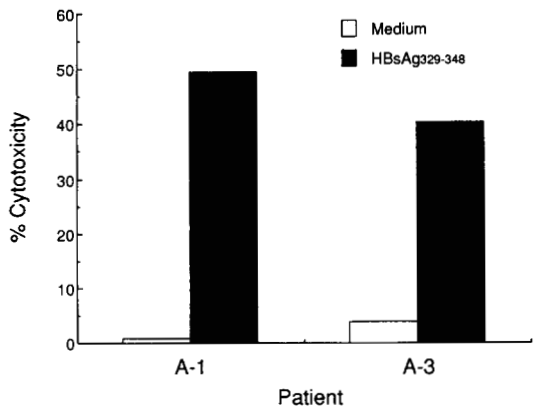

FIGURE 1. A synthetic peptide representing $HBsAg_{329-348}$ (ASARFSWLSLLVPFVQWFVG) stimulates a specific cytotoxic response in PBMC of HLA-A2 positive AVH-B patients. After stimulation with $HBsAg_{329-348}$, PBMC lines from two AVH-B patients were tested against allogeneic HLA-$A2^+$ JY cells, prepulsed with 10 μg/ml of the same peptide overnight, in a 4-h ^{51}Cr-release assay. PBMC stimulation time and E:T ratio were 20 days, 40:1 for patient A-1, and 18 days, 30:1 for patient A-3.

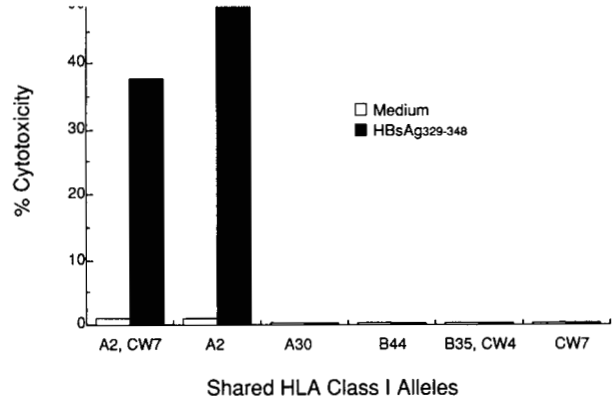

FIGURE 2. $HBsAg_{329-348}$ cytotoxic response is HLA-A2-restricted. CTL clone B3 from patient A-1, generated by stimulation with $HBsAg_{329-348}$ peptide, showed cytotoxicity against allogeneic HLA-A2-matched target cells prepulsed overnight with 10 μg/ml of the same peptide. Sharing HLA class I at other loci did not render target cells susceptible to lysis. Cytotoxicity was measured at E:T of 15:1 in a 4-h ^{51}Cr-release assay.

Results

Generation and HLA restriction analysis of $HBsAg_{329-348}$-specific CTL lines and clones

Two HLA-$A2^+$ patients with acute hepatitis (A-1 and A-3) were initially selected for analysis of the CTL response to $HBsAg_{329-348}$ (ASARFSWLSLLVPFVQWFVG), which contains two overlapping ideal HLA A2.1 allele specific binding motifs (WLSLLVPFV and LLVPFVQWFV) as described by Falk et al. (14). One of these patients (A-3) was known from previous experiments to display an HLA A2-restricted CTL response to a 10-residue HBV nucleocapsid epitope ($HBcAg_{18-27}$) that also represents an ideal HLA A2.1 allele-specific binding motif (FLPSDFFPSV). This patient was considered a potential responder to one or both of the motifs in $HBsAg_{329-348}$. Another patient (A-1), known to be a nonresponder to $HBcAg_{18-27}$, was studied for comparison.

As shown in Figure 1, $HBsAg_{329-348}$-specific CTL lines were generated from PBMC of both patients by stimulation with the peptide as described above. After 2 to 3 wk of stimulation, both patients displayed a strong cytotoxic response against a homozygous HLA $A2.1^+$ EBV cell line (JY) prepulsed with the $HBsAg_{329-348}$ peptide. Patient A-1's $HBsAg_{329-348}$ specific cell line was selected for cloning. Four clones were derived from cells plated at 1 cell/well (clone B13, B16, B17) or 0.3 cells per well (clone B3). Clone B3 was tested against a panel of allogeneic target cells partially matched with the effectors at the level of HLA class I alleles. As shown in Figure 2, by using allogeneic target cells partially matched at HLA class I with patient A-1, the cytolytic activity of clone B3 was found to be HLA-A2-restricted, as expected, due to the presence of two ideal HLA-A2.1-binding motifs in the peptide. An $HBsAg_{329-348}$-specific polyclonal CTL line derived from patient A-3 was also shown to be HLA-A2-restricted in the same manner (not shown). Because the HLA-A2 subtypes of our patients were not determined, we do not know if the CTL response to the peptides studied in this report is restricted only by the HLA-A2.1 allele or whether it extends to other HLA-A2 subtypes as well.

$HBsAg_{335-343}$ is the minimum, optimal HLA-A2-restricted CTL epitope within $HBsAg_{329-348}$

A panel of amino-terminal truncations and overlapping 9 and 10 mers derived from the $HBsAg_{329-348}$ sequence were produced to map the HLA-A2-restricted CTL epitope(s) present in this 20-residue peptide, which contains two overlapping ideal HLA-A2.1-binding motifs (Fig. 3). The HLA-A2-restricted CTL clone B17 from patient A-1, and a polyclonal CTL line 1B9 from patient A-3, derived by repetitive stimulation of the cell line shown in Figure 1 with $HBsAg_{329-348}$, were used as effector cells to establish the fine specificity of the CTL response to $HBsAg_{329-348}$. Target cells were produced by incubating an HLA-$A2.1^+$ B cell line (JY) either with the original 20-mer or with the truncated peptides.

The results indicate that only the first of the two ideal HLA-A2.1-binding motifs ($HBsAg_{335-343}$) is recognized by the CTL. Furthermore, the data demonstrate that this peptide (WLSLLVPFV) is indeed the minimal HLA-A2-restricted epitope recognized by $HBsAg_{329-348}$-stimulated CTL, because omission of the extreme amino-terminal or the extreme carboxy-terminal residue from $HBsAg_{335-343}$ abolishes recognition by the CTL (Fig. 3). The same peptide is also the optimal epitope recognized by the CTL because

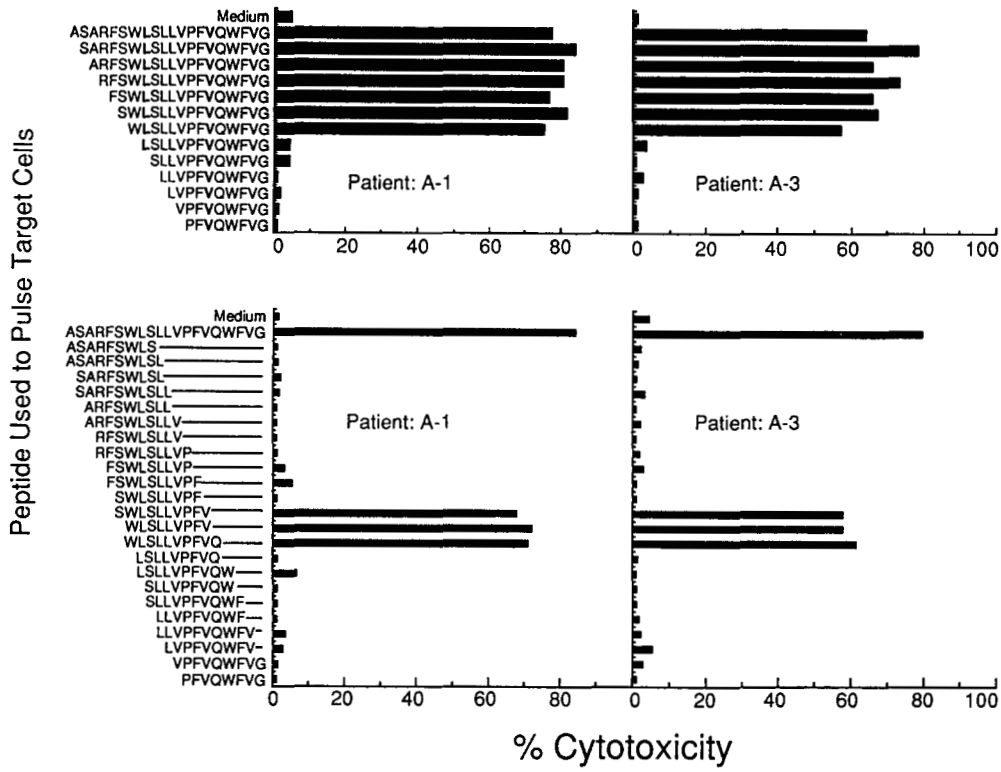

FIGURE 3. $HBsAg_{335-343}$, WLSLLVPFV, is the minimal CTL epitope recognized by CTL stimulated by $HBsAg_{329-348}$. A CTL clone from patient A-1 and a CTL cloned line from patient A-3 generated by stimulation with $HBsAg_{329-348}$ were tested against JY target cells prepulsed either with truncations (*upper panels*) or with overlapping 9 or 10 mers (*lower panels*) covering $HBsAg_{329-348}$. JY target cells were prepulsed overnight with the peptides at 10 μM final concentration in all the experiments shown.

it was able to prime target cells for killing at very low concentrations (e.g., 10^{-7} to 10^{-8} M) that were comparable to the longer peptides (e.g., $HBsAg_{334-343}$, $HBsAg_{335-344}$, $HBsAg_{329-348}$) within which it is contained (not shown).

The superiority of $HBsAg_{335-343}$ at the effector level was reiterated when the peptides were used to stimulate a CTL response in PBMC from patient A-1. As can be seen in Figure 4, although $HBsAg_{335-343}$ and its extended variants proved capable of inducing a CTL response, omission of the extreme amino- and carboxy-terminal amino acids completely abolished the ability of the peptides to stimulate a CTL response, thereby reinforcing the argument that $HBsAg_{335-343}$ is the minimal optimal HLA-A2-restricted epitope between residues 329 to 348 of HBsAg.

Analysis of the CTL response to seven ideal HLA-A2.1 binding motifs in the HBV envelope protein

A total of 7 ideal HLA-A2.1 allele-specific binding motifs, defined as peptides between 9 and 10 residues in length that contain a leucine in the second position and a valine as the carboxy-terminal residue, are present in the HBsAg region of the HBV envelope protein (Table II). Based on the foregoing results, we decided to examine the ability of these seven envelope peptides, plus the known HLA-A2-restricted HBV nucleocapsid epitope ($HBcAg_{18-27}$), to stimulate a CTL response in 12 HLA-$A2^+$ patients with acute hepatitis B (Fig. 5). For comparison, we tested six HLA-$A2^+$ patients with chronic hepatitis and six uninfected normal controls for responsiveness to the same panel of peptides.

As can be seen in Figure 5, 9 of the 12 HLA-$A2^+$ patients with acute hepatitis responded to at least one of the peptides in the panel. In contrast, none of the six HLA-$A2^+$ uninfected normal controls responded to any of the peptides after the same in vitro stimulation strategy (Fig. 5), suggesting that responsiveness to these peptides by the patients reflects in vivo priming by the corresponding HBV-derived epitopes.

Importantly, eight of the nine responders recognized multiple epitopes within the panel, indicating that the CTL response to HBV during acute hepatitis is both polyclonal and multispecific. Furthermore, there was substantial variation in the spectrum of epitopes recognized among the nine responders, with certain epitopes being recognized more frequently than others. For example, $HBcAg_{18-27}$ and $HBsAg_{335-343}$ were recognized individually by seven and eight of the nine responders, respectively, and when

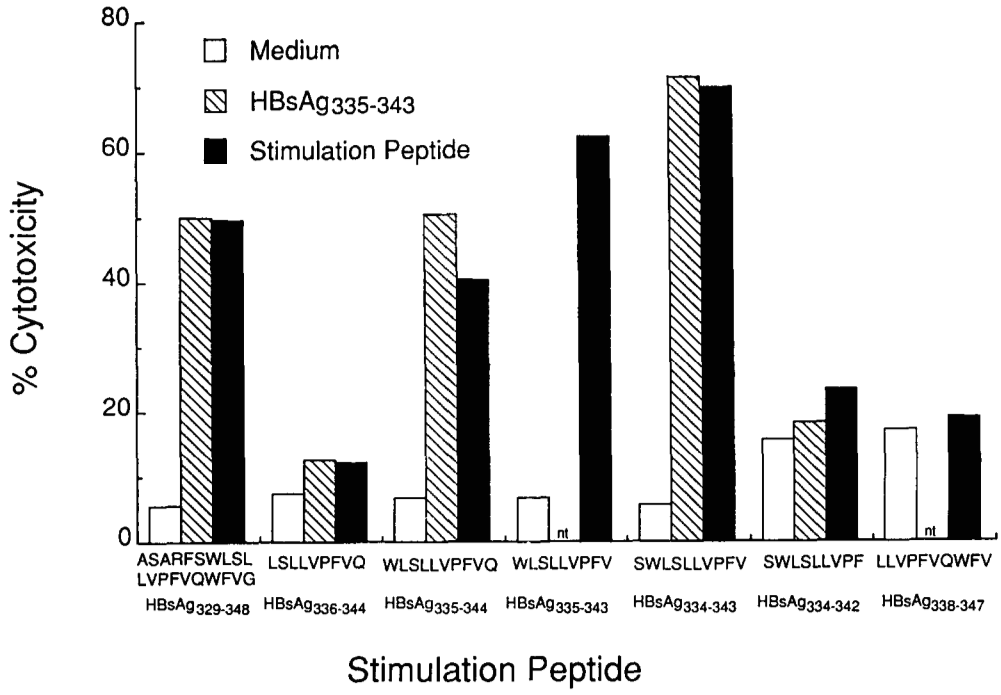

FIGURE 4. The minimal epitope within $HBsAg_{329-348}$ for in vitro CTL induction is $HBsAg_{335-343}$. Synthetic peptides representing assorted $HBsAg_{329-348}$ subunits were used at 10 μM to stimulate PBMC of patient A-1. After 2 wk of stimulation the cytotoxicity of these lines was tested at E:T of 60:1 against JY target cells prepulsed with 10 μM of the same peptide and JY target cells prepulsed with 10 μM $HBsAg_{335-343}$. Results shown represent percent lysis in a 4-h ^{51}Cr-release assay.

combined they were recognized by all nine of the responders. In contrast, $HBsAg_{348-357}$, $HBsAg_{251-259}$, and $HBsAg_{260-269}$ were recognized by only 3/9, 2/5, and 3/6 of the responders in whom they were tested. The basis for the apparent hierarchy of immunogenicity among these seven envelope peptides is not understood at present.

It is noteworthy that all nine responders subsequently became $HBsAg^-$ and their liver disease completely resolved. In contrast, all six patients with chronic hepatitis who failed to clear the virus, also failed to mount a peripheral blood CTL response to any of these epitopes (Fig. 5). It is very interesting that three of the acutely infected patients (A-10, A-11, A-12) also failed to respond to any of these peptides. Furthermore, one of the nonresponders (A-11) developed chronic active hepatitis and was still $HBsAg^+$ 13 mo after his acute illness. Although these combined data suggest a relationship between the CTL response and viral clearance, it is important to point out that nonresponder patient A-12 seroconverted to HBsAg negativity between 1 and 4 mo after disease onset. Unfortunately, patient A-10 was lost to follow-up after his first clinic visit. Thus it will be necessary to study many additional patients with an expanded panel of epitopes to determine the extent to which the HBV-specific CTL response correlates with viral clearance during acute viral hepatitis.

As indicated in Table I and Figure 5, four of the nine responders share only the HLA A2 allele with the JY target cell line used in this study (HLA-A2, B7, Cw7), demonstrating that the response to all of the peptides was HLA-A2- restricted in these individuals. Because the remaining responders also share the HLA B7 and/or Cw7 alleles present in the JY target cells in addition to A2, it is formally possible, although unlikely, that these alleles could also serve as restriction elements for these epitopes in these patients.

Binding capacity of HBsAg peptides containing HLA-A2.1-binding motif

Although we do not understand the basis for the apparent hierarchy of immunogenicity among these seven envelope peptides, factors at the level of virus sequence, HLA-A2.1-binding affinity, Ag processing, and host T cell repertoire could account for these differences. To determine whether the differential immunogenicity of the peptides was related to their relative HLA-A2.1-binding affinity, we compared the ability of the nonimmunogenic ($HBsAg_{201-210}$, $HBsAg_{338-347}$, and $HBsAg_{378-387}$) and the immunogenic ($HBsAg_{251-259}$, $HBsAg_{260-269}$, $HBsAg_{335-343}$, and $HBsAg_{348-357}$) peptides to bind HLA-A2.1 in a competitive binding inhibition assay by using an $HBcAg_{18-27}$-specific CTL clone from patient A-4. As can be seen in Figure 6, although the nonimmunogenic peptide $HBsAg_{338-347}$ shows minimal binding of HLA-A2.1, the other two nonimmunogenic peptides bind HLA-A2.1 as well as the immunogenic peptides. Indeed, nonimmunogenic

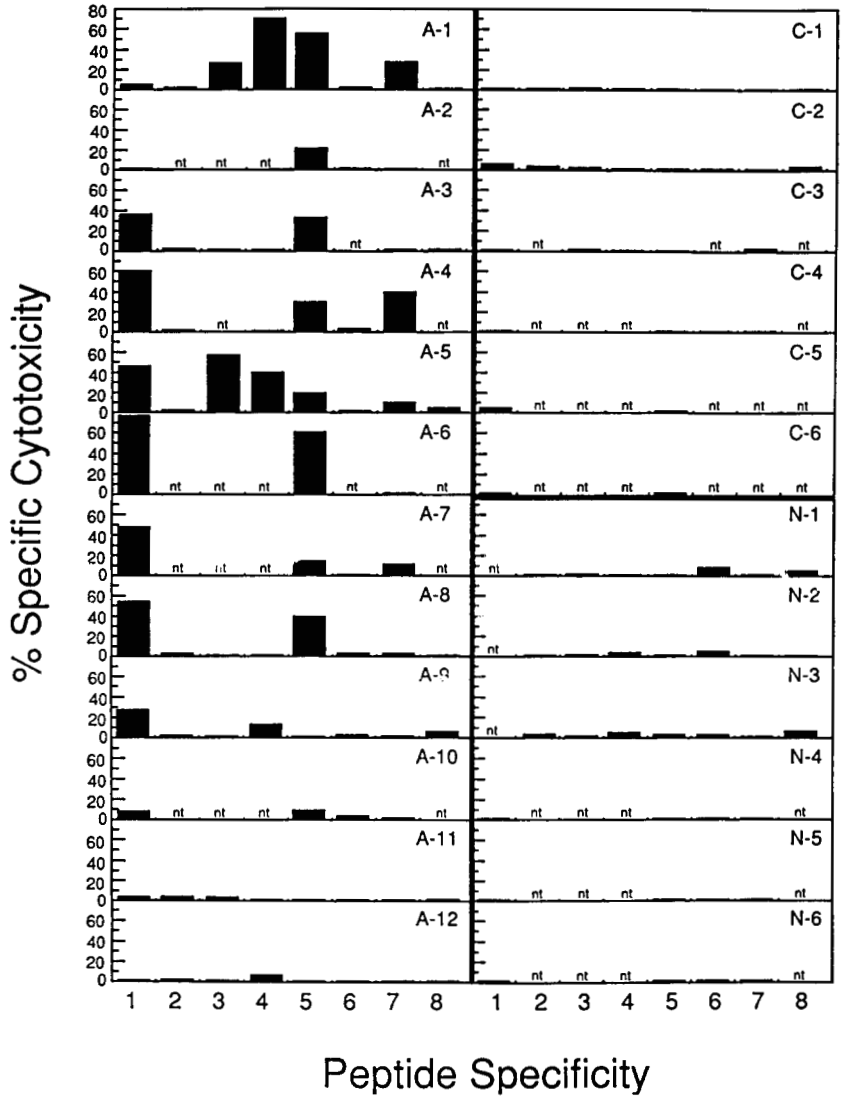

FIGURE 5. HBV specific CTL response in patients with acute hepatitis B infection, chronic hepatitis B infection, and normal subjects. PBMC from acute patients (A-1 to A-12), chronic patients (C-1 to C-6), and normal subjects (N-1 to N-6) were stimulated with the following synthetic peptides: *1*, $HBcAg_{18-27}$; *2*, $HBsAg_{201-210}$; *3*, $HBsAg_{251-259}$; *4*, $HBsAg_{260-269}$; *5*, $HBsAg_{335-343}$; *6*, $HBsAg_{338-347}$; *7*, $HBsAg_{348-357}$; *8*, $HBsAg_{378-387}$ for 2 wk as described in *Materials and Methods*, and tested in a 4-h ^{51}Cr-release assay against JY target cells prepulsed overnight with the same peptide. Peptide-specific cytotoxicity was measured by subtracting the ^{51}Cr release by JY target cells not prepulsed with peptide from the ^{51}Cr release by JY target cells prepulsed with the peptide. Results shown represent percent specific lysis in a 4-h ^{51}Cr-release assay.

$HBsAg_{378-387}$ binds to HLA-A2.1 as well as $HBsAg_{335-343}$, the most immunogenic of the envelope peptides in the panel; and $HBsAg_{201-210}$ binds HLA-A2.1 more effectively than $HBsAg_{348-357}$. These results indicate that although HLA-A2.1-binding capacity would appear to be a prerequisite for immunogenicity, it does not guarantee that a given peptide will be immunogenic.

Epitope coding sequence in circulating HBV virion DNA in acutely infected patients: correlation with CTL responsiveness to the epitopes

It is possible that nonresponse to a given epitope may reflect infection of the patient by a virus that contains a variant sequence different from the peptide used to stimulate the CTL precursor population in vitro. To investigate this possibility, we analyzed the nucleotide sequence of a region of the HBV genome that included two immunogenic epitopes ($HBsAg_{335-343}$ and $HBsAg_{348-357}$) in HBV virion DNA that we purified from the serum of 10 patients, 8 of whom were responsive to one or both epitopes whereas two (A-10 and A-12) were not. Unfortunately, serum was not available from nonresponder patient A11 for analysis.

As illustrated in Table III, we found that all of the patients, including the CTL nonresponders, were infected by viruses that expressed the precise amino acid sequence present in the prototype $HBsAg_{335-343}$ peptide used to stimulate the expansion of CTL in vitro. Therefore, nonresponse to this sequence was not due to infection by a variant virus. Because residues 335 to 343 are known to be conserved in all the published HBV sequences derived from all four HBV subtypes, as published in the GenBank-72 database, as well as in the 10 patients studied herein, we conclude that $HBsAg_{335-343}$ is an HBV group-specific CTL epitope.

The same was not true for $HBsAg_{348-357}$, however, because only 7 of the 10 patients were found to be infected by viruses that encode the prototype sequence we used for in vitro stimulation (GLSPTVWLSV). The remaining three patients (A-9, A-10, A-13) displayed a variant sequence in

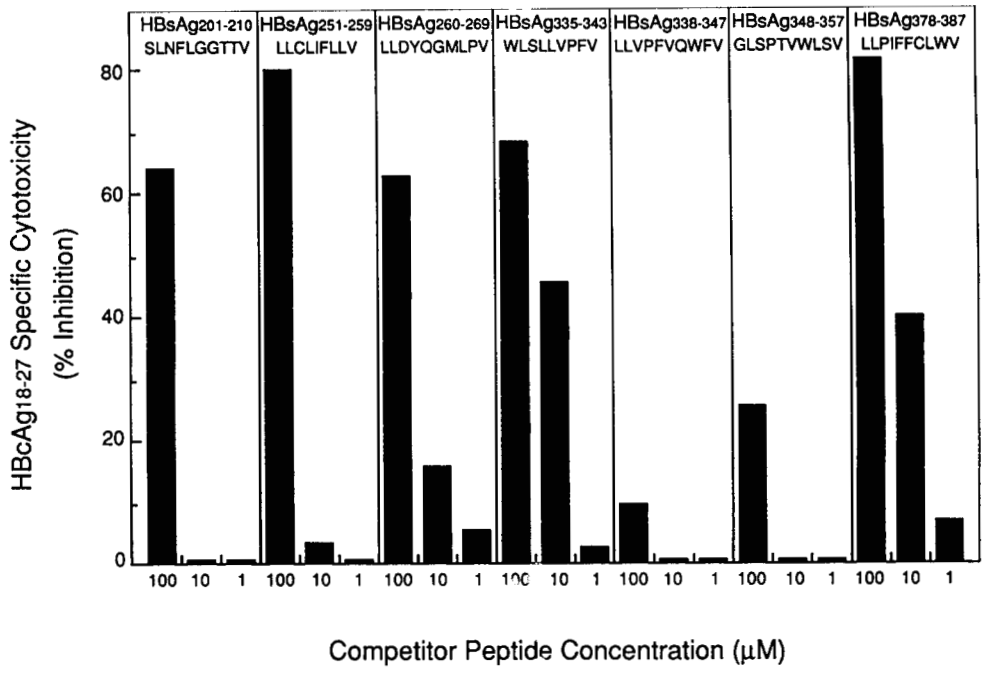

FIGURE 6. HLA-A2.1 competitive binding inhibition assay. HBsAg peptides were added to JY target cells and an $HBcAg_{18-27}$-specific CTL clone (E:T = 40:1, 3000 target cells/well) at various concentrations for 40 min before the addition of peptide $HBcAg_{18-27}$ (0.03 μM final concentration). Results shown represent percent inhibition of $HBcAg_{18-27}$-specific lysis in a 4-h ^{51}Cr- release assay.

Table III
Relationship between virus sequence and CTL response

Patient	CTL Response (% Cytotoxicity)		DNA Sequence of Infecting Virus (Prototype Sequence)
	WLSLLVPFV	GLSPTVWLSV	WLSLLVPFVQWFVGLSPTVWLSV
A-1	55	27	----------
A-4	29	40	----------
A-5	19	9	----------
A-6	61	0	----------
A-7	14	11	----------
A-8	40	3	----------
A-9	0	2	----------A
A-10	9	2	----------A
A-12	0	0	----------
A-13	14	2	----------A

which the carboxy-terminal valine was substituted by an alanine at position 357. Among the patients infected by the prototype virus, we observed CTL responders and nonresponders to $HBsAg_{348-357}$, just as we did for the response to $HBsAg_{335-343}$. On the other hand, none of the three patients infected by the variant virus displayed a CTL response to the prototype peptide. Consistent with this observation, CTL specific for the prototype $HBsAg_{348-357}$ sequence from patient A-4 did not recognize a variant peptide in which the carboxy-terminal residue was an alanine (Fig. 7) despite the fact that the HLA-A2.1-binding affinity of the variant peptide was almost as high as the prototype peptide (not shown). Interestingly, the variant peptide failed to stimulate a CTL response in vitro in patient A-9 who was infected by a virus containing the homologous sequence (not shown).

Recognition of endogenously synthesized HBsAg by peptide-specific CTL

The ability of $HBsAg_{335-343}$- and $HBsAg_{348-357}$-specific CTL to recognize endogenously synthesized Ag was examined by measuring their ability to lyse target cells that had been infected with two groups of recombinant vaccinia viruses which encode the large, middle, and major envelope polypeptides derived from cloned HBV genomes of either the ayw or the adw subtypes of HBV. Both $HBsAg_{335-343}$- and $HBsAg_{348-357}$-specific CTL from patients A-1 and A-4 were able to lyse recombinant vaccinia virus-infected target cells that synthesize all three of the HBV envelope proteins (Fig. 7). This indicates that both of these synthetic peptides represent epitopes that are generated by the endogenous processing of the large, middle, and major HBV envelope polypeptides within infected cells.

Importantly, $HBsAg_{335-343}$-specific CTL could lyse targets that were infected by both sets of recombinant vaccinia viruses with equal efficiency, whereas the $HBsAg_{348-357}$-specific CTL lysed the ayw-infected target cell panel much more efficiently than the adw targets (Fig. 7). To examine the molecular basis for this observation, the nucleotide sequence of a PCR-amplified fragment that spans envelope codons 335 to 357 was determined for the HBV-derived coding regions present in both sets of recombinant

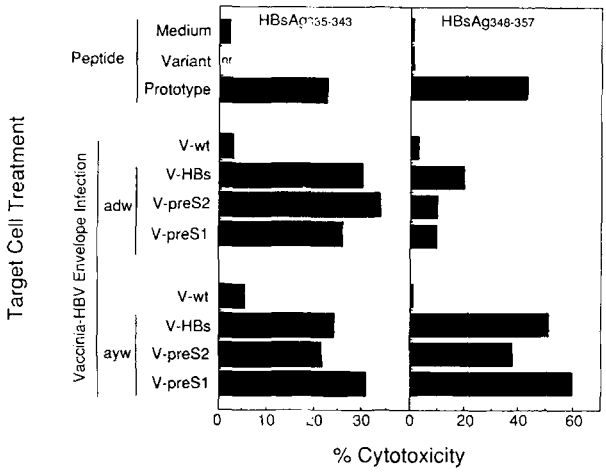

FIGURE 7. The CTL response to $HBsAg_{335-343}$ and $HBsAg_{348-357}$ are group-specific and subtype-specific, respectively. An $HBsAg_{335-343}$-specific CTL line (patient A-1) and an $HBsAg_{348-357}$-specific CTL line (patient A-4) were generated by stimulation with peptide sequences WLSLLVPFV and GLSPTVWLSV, respectively. CTL were incubated with ^{51}Cr-labeled JY target cells that had been preincubated either with media, with the inducing peptide or (in the case of $HBsAg_{348-357}$) with a variant peptide (GLSPTVWLSA). CTL were also incubated with ^{51}Cr-labeled JY target cells that had been infected with a panel of six recombinant vaccinia viruses that express the HBV major (*V-HBs*), middle (*V-preS2*), and large (*V-preS1*) envelope polypeptides derived from ayw and adw HBV genomes. Wild-type vaccinia viruses (*V-wt*) were used as controls. The $HBsAg_{335-343}$-specific CTL line (*right panel*) was used at an E:T = 40:1. The $HBsAg_{348-357}$-specific CTL line (*left panel*) was used at an E:T = 3:1. Results shown represent percent lysis in a 4-h ^{51}Cr-release assay.

vaccinia-HBV expression vectors. Compatible with the cytotoxicity results (Fig. 7, Table III), the deduced amino acid sequences of the ayw- and adw-derived HBV-coding regions in the vaccinia vectors were identical with the amino acid sequence of peptide $HBsAg_{335-343}$ used for in vitro CTL stimulation in this study (WLSLLVPFV). Similarly, the ayw-derived coding region contained exactly the same sequence as peptide $HBsAg_{348-357}$ (GLSPTVWLSV). Importantly, both patients (A-1, A-4) were infected by viruses that encode precisely the same sequences (Table III). In contrast, envelope codon 357 in the recombinant vaccinia viruses containing the adw-derived coding region was an alanine (GLSPTVWLSA).

In keeping with these results, the published amino acid sequences for all HBV subtypes (GenBank-72 database) are identical between residues 335 and 356 of HBsAg. In contrast, position 357 is a valine in all subtypes except adw where it alternates between valine in some isolates and alanine in others. Based on this information, the current results suggest that the $HBsAg_{335-343}$-specific CTL response should be able to recognize all known HBV isolates (group-specific), and that the $HBsAg_{348-357}$-specific CTL response will only recognize a subset of viruses that encode a valine residue in envelope codon 357 (subtype-specific).

Discussion

In the current study we used synthetic peptides to expand the peripheral blood, $CD8^+$, HBsAg-specific cytotoxic T cell population in patients with acute and chronic hepatitis, as we have reported previously in studies of the HBV nucleocapsid-specific CTL response (8–12). This in vitro stimulation strategy expands Ag specific CTL populations that have been primed in vivo inasmuch as we have shown, here and elsewhere, that the peptide stimulated CTL can efficiently recognize targets that express endogenous Ag and because they are not induced in uninfected normal controls (8–12).

To begin our analysis of the HLA-A2-restricted CTL response to the HBV envelope protein we used a 20-residue peptide ($HBsAg_{329-348}$) containing two overlapping ideal HLA-A2.1 motifs (WLSLLVPFV and LLVPFVWQFV). The CTL response proved to be focused exclusively on the first motif ($HBsAg_{335-343}$). It was HLA-A2-restricted and capable of recognizing endogenously synthesized Ag derived from all three of the HBV envelope proteins of two independent HBV subtypes (ayw and adw), which share a common 226-amino acid HBsAg domain that contains the $HBsAg_{335-343}$ epitope. Because the amino acid sequence of residues 335 to 343 was identical in all of our patients (Table III) and because these residues are invariant among all known HBV isolates (GenBank-72 database), the current data indicate that the $HBsAg_{335-343}$-specific CTL response is HBV group-specific.

We confirmed the differential immunogenicity of these two peptides by demonstrating that peptide $HBsAg_{335-343}$ itself was also able to stimulate a strong CTL response in vitro, whereas the overlapping peptide $HBsAg_{338-347}$ could not; and we demonstrated that the potency of these two peptides correlated with their relative binding affinity for HLA-A2.1 (Fig. 6). Encouraged by these results, we extended our studies to all seven of the ideal HLA-A2.1-binding motifs present in the entire HBV envelope protein.

In a study involving 12 $HLA\text{-}A2^+$ patients with acute hepatitis we determined that four of the motifs were able to stimulate a CTL response and three could not (Fig. 5). It was interesting that all 9 of the 12 acutely infected patients who developed a polyclonal, multispecific CTL response to HBV cleared the virus and completely resolved their liver disease, whereas none of the 6 $HLA\text{-}A2^+$ patients with chronic hepatitis produced a detectable CTL response to any of the epitopes tested (Fig. 5). Based on these observations we suggest that the virus-specific CTL response plays an important role in viral clearance and,

perhaps, in the pathogenesis of liver disease during HBV infection.

In support of this hypothesis, we observed that one of the three acutely infected nonresponders subsequently developed chronic active hepatitis. However, one of the other nonresponders ultimately cleared the virus within a few months after the onset of disease. Obviously, the significance of these contrasting observations depends on the completeness of the panel of epitopes studied and the sensitivity of the CTL detection system employed. Indeed, it is possible that both of these "nonresponders" may have produced a response to currently unknown epitopes that were not used in the current study. Thus it is imperative to continue to identify additional HLA class I restricted CTL epitopes in HBV so that a more comprehensive analysis can be performed to assess the role played by the CTL response in viral clearance during HBV infection. If CTL nonresponse during clinically acute infection proves to be a marker to identify patients who are destined to become chronically infected by HBV, it may have clinical implications with respect to selection of patients for early antiviral therapy.

The molecular basis for the differential immunogenicity of the seven HBV envelope peptides is not immediately evident from a comparison of their sequences. It could be due to viral factors such as amino acid sequence variability at these loci in the viral genomes that infect different patients, or differences in the relative HLA-A2.1-binding affinity of the various peptides. Alternatively, variable host factors at the level of Ag processing, HLA-A2 subtype variability, and the T cell repertoire could account for the differential immunogenicity of the predicted HLA-A2.1-binding motifs.

We addressed the issue of differential HLA-A2.1-binding affinity by monitoring the ability of the seven envelope peptides to compete with the binding of an unrelated HLA-A2-restricted nucleocapsid epitope ($HBcAg_{18-27}$) to a homozygous HLA-A2.1$^+$ B cell line (JY). As shown in Figure 6, all four immunogenic peptides and two of the three nonimmunogenic peptides bind to the HLA-A2.1 molecule, but with widely (more than 100-fold) variable efficiencies that did not correlate with their relative immunogenicity. Importantly, the only peptide that did not bind to HLA-A2.1 in this assay ($HBsAg_{338-347}$) was nonimmunogenic. For the other two nonimmunogenic peptides, however, the HLA-A2.1-binding affinity was as high or higher than some of the immunogenic peptides. Thus, although the capacity of a peptide to bind to this class I molecule seems to be required for immunogenicity, it does not guarantee it, indicating that additional factors at the level of Ag processing and the T cell repertoire probably play an important role in determining which HLA-A2.1-binding peptides within a viral protein are able to induce a CTL response. This is consistent with recent results showing that immunodominance is not simply determined by the primary amino acid sequence, but is a function of the context of the epitope within the protein such that intramolecular competition for cellular processing and HLA binding functions greatly influence the selection of epitopes to which a CTL response is made (24). It is also consistent with the observation that the more rapidly proteins are degraded, the more effectively they are presented to class I-restricted CTL (25).

It is also possible that amino acid sequence heterogeneity within the infecting virus may contribute to the differential CTL responsiveness observed in individual patients. That is to say, nonresponse to a given HBV epitope may reflect infection by a variant of HBV in which the epitope region differs from the prototype sequence used to synthesize the peptide. In this situation, a CTL response to the prototype epitope sequence used for in vitro stimulation would not be induced in vivo. We examined this possibility by PCR amplification and sequence analysis of the serum derived virion DNA corresponding to HBsAg residues 335 through 357 in 10 patients whose CTL response to epitopes $HBsAg_{335-343}$ and $HBsAg_{348-357}$ had been determined, and for which a number of responders and nonresponders had therefore been identified (Table III). Because the study group included patients who responded to one or more of the other envelope epitopes, their failure to respond to either of these two epitopes could not have been due to some generalized immunologic nonresponsiveness to epitopes containing the HLA-A2.1-binding motif and might have therefore been due to infection by a viral variant that was mutated within $HBsAg_{335-343}$ or $HBsAg_{348-357}$.

Importantly, we found that all of the patients, including the CTL nonresponders, were infected by viruses that expressed the precise amino acid sequences present in peptide $HBsAg_{335-343}$ (WLSLLVPFV) that we used to stimulate the CTL response (Table III). These results, together with the excellent binding capacity of this peptide for the HLA-A2.1 molecule (Fig. 6), suggest that the variable CTL response to this epitope is probably a function of host rather than viral factors. The virus is not entirely neutral in the process of CTL epitope selection, because we demonstrated that three patients who did not respond to stimulation with the prototype peptide $HBsAg_{348-357}$ (GLSPTVWLSV) were actually infected by a viral variant whose sequence contains an alanine substitution at position 357 (Table III).

In a related study, we showed that HLA-A2-restricted CTL that could lyse target cells that had been pulsed with the prototype peptide $HBsAg_{348-357}$, were unable to kill target cells that had been pulsed by a variant peptide containing the alanine 357 substitution (Fig. 7), even though the variant peptide could bind to HLA-A2 as well as the prototype peptide. Furthermore, these CTL were only minimally able to kill target cells that had been infected by recombinant vaccinia viruses (adw subtype) that expressed

the variant sequence (Fig. 7). In addition, one of the patients who was infected by a virus containing the variant sequence (A-9) failed to generate a CTL response after in vitro stimulation with the variant peptide. Thus, it would appear that the conservative substitution of an alanine for a valine as the carboxy-terminal anchor residue may have paradoxically affected the ability of the peptide to be recognized by the CTL receptor more than it affected its binding to the HLA-A2 molecule. Although the molecular basis for this very interesting phenomenon is not yet defined, the results clearly demonstrate that virus sequence variability from patient to patient can greatly influence the diversity of the CTL repertoire that is activated in response to the infection. Taken to the extreme, it is formally possible that global CTL nonresponse to HBV could occur in the setting of infection with an extensively mutated viral genome. We think that this is highly unlikely, however, in view of the polymorphism of the major histocompatibility complex in man, and because we have already identified a large number of CTL epitopes even though we have only studied two of the four viral proteins and a single restriction element.

We suggest that the diversity and vigor of the CTL response in acutely infected patients should minimize the chances that inactivating mutations within any one of these CTL epitopes will confer a selective survival advantage for a mutant virus. Certainly, if a mutation were to occur in a critical residue within an epitope against which a given patient's CTL response is entirely focused, CTL escape mutants could emerge. Conceivably this might occur in chronically infected patients, or perhaps in those acutely infected patients who do not respond vigorously to the virus. However, if for some reason a mutant virus can escape recognition in the original host the mutation should be irrelevant to subsequent hosts unless they have exactly the same HLA haplotype as the proband; a very rare situation indeed. Furthermore, because our data indicate that all three of the HBV envelope proteins are good targets of the HBsAg specific CTL response (Fig. 7), even mutant viruses that lose the ability to synthesize two of these three envelope proteins in their entirely should not escape CTL recognition, because the epitopes would still be generated by processing of the remaining protein.

In this context, it is also noteworthy that epitopes $HBsAg_{251-259}$ and $HBsAg_{260-269}$ are present within an important topogenic sequence involved in the transmembrane orientation of HBsAg (26). This is important because inactivating mutations in either of these epitopes might actually be deleterious for the virus inasmuch as this topogenic sequence is thought to be required for virus particle assembly. In fact, the same might be true for all of the epitopes described in this report, except $HBsAg_{348-357}$, because their amino acid sequences are entirely conserved among all known HBV isolates (GenBank-72 database), suggesting that mutations in these epitopes are not tolerated by the virus. According to this line of reasoning, generation of a CTL response to these particular domains of the HBV envelope protein might actually yield a double benefit to the host; first, by destroying the infected cells and, second, by precluding the emergence of viable mutants.

The identification of these CTL epitopes in HBsAg provides the first insight into the HLA class I-restricted HBV envelope-specific CTL repertoire during acute HBV infection. Before this study, Barnaba and colleagues (27) had isolated HLA-A3-restricted intrahepatic CTL clones specific for preS2 Ag residues 120 to 134 in a single patient with chronic active hepatitis B. Additionally, Jin and colleagues (28) described an HLA-A11-restricted CTL response to residues 21 to 28 of the preS1 Ag in a single uninfected individual who had been vaccinated with a plasma-derived HBV envelope protein vaccine. It is noteworthy that both of these previously described CTL clones were generated by stimulation with intact viral proteins rather than synthetic peptides or endogenously synthesized Ag, suggesting that internalized exogenous Ag can enter the HLA class I processing pathway within the cell. The extent to which this process occurs in hepatocytes and APC during HBV infection in vivo is currently unknown, so the relevance of these very interesting observations to HBV pathogenesis is currently unclear.

Finally, as we observed for the $HBcAg_{18-27}$-specific response (11), CTL that recognize these HBsAg epitopes were not detectable in HLA-$A2^+$ patients with chronic hepatitis. It is possible that this reflects the expression of HLA-A2 subtypes in these patients that might not be able to bind the ideal HLA-A2.1 motif, or infection by mutant viruses lacking these HLA-A2 binding motifs, or the complete absence of an HBV-specific CTL response, or a skewing of the response to epitopes that are recognized poorly or not at all by patients who successfully clear the virus from the liver. Alternatively, these patients may generate a perfectly normal CTL response which, for unknown reasons, is limited to the intrahepatic compartment and does not spill over into the peripheral blood. Future studies will be necessary to examine the relative contribution of each of these factors to peripheral blood CTL nonresponse to HBV during chronic hepatitis. Depending on the outcome of these studies it is possible that immunotherapeutic augmentation of the HBV-specific CTL response might lead to viral clearance in patients with chronic hepatitis, assuming that appropriately selected synthetic CTL epitopes can be rendered immunogenic in vivo.

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