

## Cytotoxic T Lymphocyte Response to a Wild Type Hepatitis B Virus Epitope in Patients Chronically Infected by Variant Viruses Carrying Substitutions within the Epitope

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### Summary

Mutations that abrogate recognition of a viral epitope by class I-restricted cytotoxic T lymphocyte (CTL) can lead to viral escape if the CTL response against that epitope is crucial for viral clearance. The likelihood of this type of event is low when the CTL response is simultaneously directed against multiple viral epitopes, as has been recently reported for patients with acute self-limited hepatitis B virus (HBV) infection. The CTL response to HBV is usually quite weak, however, during chronic HBV infection, and it is generally acknowledged that this is a major determinant of viral persistence in this disease. If such individuals were to produce a mono- or oligospecific CTL response, however, negative selection of the corresponding mutant viruses might occur. We have recently studied two HLA-A2-positive patients with chronic hepatitis B who, atypically, developed a strong HLA-A2-restricted CTL response against an epitope (FLPSDFFPSV) that contains an HLA-A2-binding motif located between residues 18-27 of the viral nucleocapsid protein, hepatitis B core antigen (HBcAg). These patients failed, however, to respond to any of other HLA-A2-restricted HBV-derived peptides that are generally immunogenic in acutely infected patients who successfully clear the virus. Interestingly, DNA sequence analysis of HBV isolates from these two patients demonstrated alternative residues at position 27 (V → A and V → I) and position 21 (S → N, S → A, and S → V) that reduced the HLA and T cell receptor-binding capacities of the variant sequences, respectively. Synthetic peptides containing these alternative sequences were poorly immunogenic compared to the prototype HBc18-27 sequence, and they could not be recognized by CTL clones specific for the prototype peptide. While we do not know if the two patients were originally infected by these variant viruses or if the variants emerged subsequent to infection because of immune selection, the results are most consistent with the latter hypothesis. If this is correct, the data suggest that negative selection of mutant viral genomes might contribute to viral persistence in a subset of patients with chronic HBV infection who express a narrow repertoire of anti-HBV CTL responses.

**H**LA class I-restricted, anti-viral cytotoxic T cells recognize short viral peptides that are generated by the intracellular processing of endogenously synthesized viral antigen within infected cells (1, 2), and are expressed at the cell surface in the binding groove of selected HLA class I molecules (3-7). Peptide binding to HLA class I molecules is dependent on interactions between conserved MHC residues and main chain atoms at both peptide termini (8-13), as well as selective interactions between allele-specific MHC pockets,

formed by polymorphic MHC residues, and side chains of peptide anchor residues, pointing towards the floor of the HLA groove (14-17). Residues of the MHC bound peptide particularly accessible to TCR recognition are located in the central portion of the antigenic sequence that can face outwards from within the MHC binding groove (18).

Single amino acid substitutions of MHC anchor residues or TCR contact sites have been shown to abrogate CTL responses in vitro by inhibiting either HLA binding or TCR

recognition of the peptide (19). By extension, therefore, it is conceivable that mutations that destroy CTL epitopes may permit viral escape and the establishment of viral persistence by the variant virus in vivo. Indeed, this has been shown to occur in transgenic mice that express a lymphocytic choriomeningitis virus-specific T cell receptor and produce a monoclonal cytotoxic response that is focused on a single CTL epitope (20).

In contrast to these TCR transgenic mice, the CTL response during human viral infections is usually polyclonal and multispecific which virtually precludes the emergence of escape mutants (21–26). Nonetheless, HIV genomes containing mutations within an HLA-B8-restricted HIV gag CTL epitope have been shown to emerge in an HIV-seropositive donor (27), and a dominant EBV genotype that contains an inactivating substitution within an immunodominant HLA-A11-restricted CTL epitope has been reported in a high HLA-A11 frequency human population (28). Thus, it is possible that functionally monospecific CTL responses to certain viruses that create the conditions required for the negative selection of CTL escape mutants may occur during infection by pathogenic human viruses.

During acute hepatitis B virus (HBV)<sup>1</sup> infection most patients develop a strong, polyclonal CTL response against multiple epitopes in the viral envelope, nucleocapsid, and polymerase proteins, that is vigorous enough to be detected in the peripheral blood (26, 29–31; Rehmann, B., J. Person, A. Redeker, P. Fowler, M. Brown, B. Moss, A. Sette, and F. V. Chisari, manuscript in preparation). In contrast, the peripheral CTL response to HBV is usually not detectable in chronically infected patients (26, 30). In the present study we describe two patients with chronic HBV infection who displayed a vigorous HLA-A2-restricted CTL response against a prototype nucleocapsid epitope located between residues 18–27 of HBcAg (32). Interestingly, these patients were infected by viruses that code for poorly immunogenic variants of the prototype epitope that are also inefficiently recognized by CTL with prototype HBc18–27 epitope specificity. The implications of these findings with respect to mechanisms of virus persistence and disease pathogenesis are discussed.

## Materials and Methods

**Patients.** Four patients with acute hepatitis type B, nine patients with chronic HBV infection, and four healthy subjects were studied. The diagnosis of acute hepatitis B was based on the finding of elevated values of serum alanine aminotransferase (ALT) activity (at least 10 times the upper limit of the normal), associated with the detection of IgM antibodies against hepatitis B core antigen in the serum, and the recent onset of jaundice and other typical symptoms of acute hepatitis. All acute patients recovered completely from the illness with normalization of transaminase and clearance of hepatitis B surface antigen from the serum. The diagnosis of chronic hepatitis type B was based on biochemical and histological findings. No patient was receiving corticosteroid or antiviral drugs. All were antibody negative to HIV, hepatitis C, and delta viruses.

<sup>1</sup> Abbreviations used in this paper: ALT, alanine aminotransferase; EBV-B, EBV-transformed B; HBV, hepatitis B virus; r, recombinant.

**DNA Preparation.** For the amplification of circulating HBV-DNA genomes, sera (100  $\mu$ l) were heated at 100°C for 30 min, centrifuged at 12,000 g for 30 min, and 5  $\mu$ l of the supernatants were directly used for PCR. Total cell DNA was extracted from fresh PBMC and liver infiltrating T cells of HBV-infected patients by the proteinase K method. Pellets of 1–5  $\times$  10<sup>6</sup> cells were lysed in 500  $\mu$ l of 10 mM Tris, pH 8, 10 mM EDTA, 10 mM NaCl, and 0.5% SDS. 2  $\mu$ l of 20 mg/ml proteinase K were immediately added and the lysate was incubated at 56°C for 3 h. After phenol/chloroform extraction and ethanol precipitation, DNA pellets were redissolved in sterile distilled water, and stored at 4°C. 1  $\mu$ g of DNA was used in each amplification. Purification of DNA from formaldehyde-fixed and paraffin-embedded liver tissue was performed according to Wright et al. (33) with minor modifications. Briefly, 5- $\mu$ m-thick liver biopsy sections were extracted twice with mixed xylenes and washed twice in ethanol. Deparaffinated liver tissue was then resuspended in 100  $\mu$ l of lysis buffer (see above) containing 200  $\mu$ g/ $\mu$ l of proteinase K and incubated at 37°C overnight. DNA was then extracted as already described and one third of each preparation was used for amplification.

**PCR Protocol.** DNA was amplified in 100  $\mu$ l of a reaction buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 100  $\mu$ g/ml of gelatin, and 20 pM of each primer. Samples were subjected to 40 cycles of amplification in a DNA thermal cycler (Perkin Elmer Corp., Norwalk, CT). The cycling conditions were 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C. The primers core 1: 5'-ACTAGGAGGCTGTAGGCATAAATTGGTCT-3' (1774–1802), core 2: 5'-GCAGTATGGTGAGGTGAACA-3' (2063–2044), core 3: 5'-TCACCTCACCATACTGCACT-3' (2046–2066), core 4: 5'-TTCCCGAGATTGAGATCTTCTGCGACGCGGCGATTGAGA-3' (2442–2404) were derived from the precore-core region of HBV genome genotype D (34).

**Cloning and Sequencing of PCR Products.** PCR products were treated with the Klenow fragment of the DNA polymerase I (Pharmacia, Uppsala, Sweden) to remove single base 3' overhangs, phosphorylated by T4 polynucleotide kinase (Pharmacia), phenol/chloroform extracted, purified on a Sephacryl S 200 micro spin column, and cloned into a pUC18 vector at the SmaI site. Several clones for each ligation were sequenced using a standard dideoxy method with a fluorescent M13 primer according to manufacturer instructions (Autoread Sequencing kit; Pharmacia). For direct sequencing of PCR products the amplification reactions were performed using a biotinylated core 1 primer. Amplification products were separated in a 2% agarose gel, the 289-bp length fragments extracted with a silicagel suspension (Quiaex gel extraction; Diagen, Dusseldorf, Germany) and the biotinylated strand immobilized on streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin; Dynal, Oslo, Norway). The single-strand template was annealed at 68°C for 5 min with 5 pM of the fluorescein-labeled sequencing primer (5'-GCAGTATGGTGAGGTGAACA-3') in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl<sub>2</sub>, and bovine serum albumine (0.1 mg/ml). The reactions were cooled at 55°C and incubated for 10 min. After a further incubation for 3 min at 37°C, 3 U of T7 DNA polymerase (Pharmacia), 6 mM dithiothreitol, and 45 mM NaCl were added. Reactions from both plasmid DNA and single-strand immobilized templates were then divided into four portions, mixed with one of the four dideoxy nucleotides, and incubated for 3 min at 37°C. An equal volume of deionized formamide containing Blue dextran (5 mg/ml) was added and the samples were loaded onto a 0.5-mm-thick 6% polyacrylamide-7 M urea gel and run on A.L.F. DNA Sequencer (Pharmacia).

**Synthetic Peptides and Antigens.** The complete panel of peptides corresponding to the HBV nucleocapsid region 18–27 containing

amino acid substitutions identified in natural HBV variants infecting two patients with chronic hepatitis B were purchased from Chiron Mimotopes (Clayton, Victoria, Australia). The panel of peptides (9- or 10-amino acids long) corresponding to the HBV sequences containing the A2-binding motif were kindly provided by Cytel (San Diego, CA). They were routinely >90% pure after reverse-phase high pressure liquid chromatography.

Recombinant (r)HBcAg was obtained from bacterial extracts of *Escherichia coli* as previously described (35). Tetanus toxoid protein was purchased from Connaught Laboratories (Swiftwater, PA).

**Generation of HBV-specific CTL.** Polyclonal CTL lines were produced as previously described (29, 30). Briefly, PBMC were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation and resuspended at  $4 \times 10^6$  cells/ml in RPMI 1640 containing 10% AB serum. Cells were stimulated with the different peptides in the presence of 1  $\mu$ g/ml of rHBcAg (Biogen, Geneva, Switzerland) in a 96-well plate (Falcon Labware, Oxnard, CA). In selected experiments, tetanus toxoid (1  $\mu$ g/ml) instead of rHBcAg was used as an alternative source of helper effect. rIL-2 (20 U/ml; Hoffmann La Roche, Basel, Switzerland) was added on day 4 of culture and the cytotoxic assays were performed on day 7.

**Production of HBc18-27-specific CTL Clones.** Oligoclonal CTL lines were generated from HBc18-27-specific polyclonal lines by limiting dilution at 1, 10, and 100 cells/well. Cells were plated in the presence of irradiated (7,000 R) HLA A2 matched EBV-transformed B (EBV-B) cell lines ( $1 \times 10^5$ /ml), allogeneic irradiated (5,000 R) PBMC ( $1 \times 10^6$ /ml), the HBc18-27 peptide (100 nM) and PHA (1:200; GIBCO BRL, Gaithersburg, MD). Res-

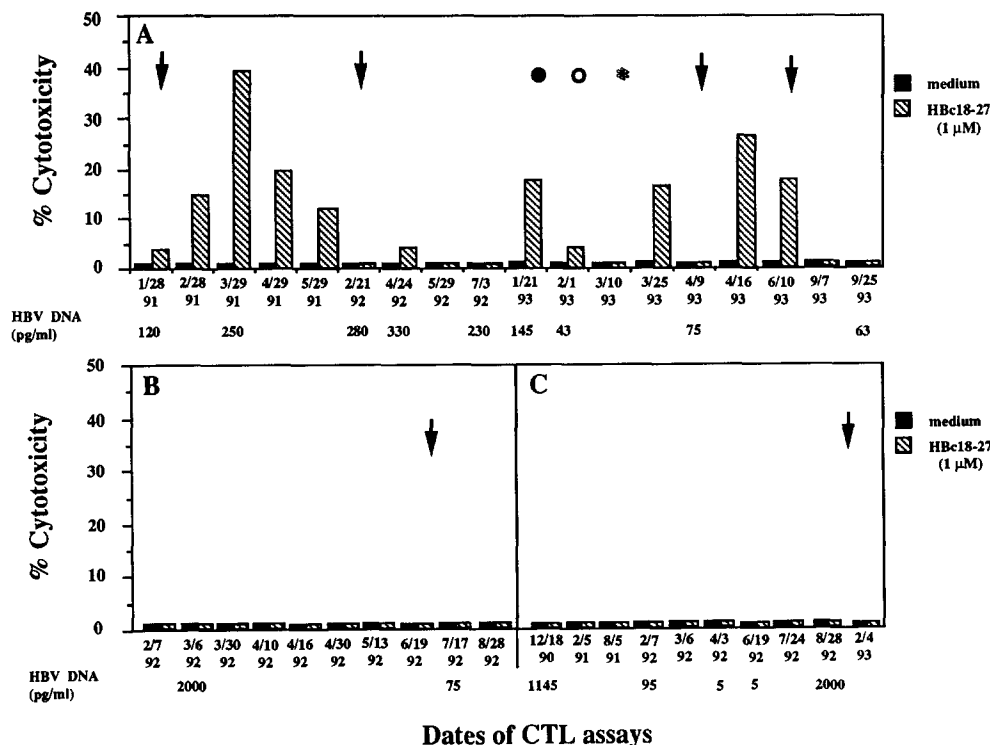
titulation was carried out every 10 d. HBc18-27-specific CTL lines were then recloned at 0.5 cells/well with the same protocol.

**Cytotoxicity Assays.** CTL lines and clones were tested for their cytolytic activity against autologous or allogeneic HLA-A2-positive EBV-B cells as targets, labeled with 100  $\mu$ Ci  $^{51}$ Cr ( $\text{Na}^{51}\text{CrO}_4$ ; Amersham International, Buckinghamshire, England) for 1 h at 37°C. Target cells were then washed twice, diluted in RPMI containing 10% FCS, and incubated for 1 h with different concentrations of the appropriate peptide, before co-culture with effector CTL at different E/T ratios in 96-well round bottomed plates. The amount of  $^{51}$ Cr released in the culture supernatants was quantitated after 4 h of incubation and percent cytotoxicity was determined using the following formula:  $(E - M/D - M) \times 100$ , where E is the experimental  $^{51}$ Cr release (cpm); M is the  $^{51}$ Cr released in the presence of culture medium (which ranged between 15 and 25% of total releasable counts); D is the total  $^{51}$ Cr released in the presence of 10% Triton X.

**HLA-A2.1 Binding Assay.** The quantitative assay used for HLA-A2.1-binding peptides based on the inhibition of binding of a radiolabeled standard peptide to detergent-solubilized HLA molecules is described elsewhere (36).

## Results

**An Efficient CTL Response against the HLA-A2-restricted Epitope HBc 18-27 Is Expressed by Some Chronic HBV Patients.** In the present study we analyzed the HBc 18-



**Figure 1.** Long-term follow-up of the CTL response against the HLA-A2-restricted epitope HBc18-27. A illustrates the CTL response displayed by one of the two chronic patients infected by the HBc 21/27 variant virus, whereas B and C illustrate the results obtained in two representative HLA-A2-positive chronic patients infected by prototype HBc18-27 virus. Chronic non-responder patients did not express significant levels of CTL activity even when peptide stimulation was performed in the presence of tetanus toxoid as an additional source of helper activity, despite the fact that this strategy proved effective in potentiating the peptide stimulatory capacity in several experiments performed in HLA-A2-positive acute and chronic peptide-responder patients (data not shown). Concentrations of serum HBV-DNA detected at different time points by a commercially available hybridization kit (HBV Chronic, Eurospital, Trieste, Italy) are also illustrated. Arrows, black circle, asterisk, and empty circle indicate the dates when virus sequencing was performed from DNA extracted from serum, liver tissue, PBMC and intra-hepatic T cells, respectively.

27-specific CTL response in nine HLA-A2-positive patients with chronic HBV infection, two of whom repeatedly displayed a significant CTL response against HBc 18-27 after 1 wk of peptide stimulation, despite their inability to clear HBV infection. In one of these two responders (patient 1) the CTL response was repeatedly examined over a time period of more than 3 yr, showing wide fluctuations of the cytotoxic activity from negative to strongly positive responses (Fig. 1 A). This finding contrasts with the consistent lack of CTL responsiveness in the majority of HLA-A2-positive patients with chronic hepatitis (reported in references 26, 30; and illustrated in Fig. 1, B and C).

*Amino Acid Substitutions within the CTL Epitope HBc 18-27 Are Present in HLA-A2-positive Chronic HBV Patients.* To investigate the basis for viral persistence despite CTL responsiveness in these two patients, the entire precore and core genes of 49 serum-derived viral genomes were amplified and sequenced. 100% (49/49) of the genomes sequenced from these two patients displayed amino acid substitutions at HBcAg positions 21 and 27 (Table I) relative to the published HBV-DNA sequences of the prototype genotype D (which, together with genotype A, is prevalent in Western Europe and North America; 34, 37) and to HBV DNA sequences obtained from 10 HLA-A2-negative patients with chronic hepatitis B derived from our geographical area (data not shown). In patient 1, serine 21 and valine 27 were consistently substituted by asparagine and alanine, respectively, at all time points studied over a 4-yr period (1990, 1992, and 1993; Fig. 1 A). Patient 2 was studied only once, but his

virus population was dominated (17/18 sequenced clones; 94%) by a variant that displayed an alanine for serine substitution at position 21, and isoleucine for valine at position 27, while the remaining clone contained a serine to valine substitution at position 21, and a valine to isoleucine at position 27 (Table 1). Similarly, all viral genomes isolated from liver tissue, PBMC, and an intrahepatic lymphocyte T cell line in patient 1 displayed asparagine 21 and alanine 27 substitutions (not shown).

In contrast, the HBcAg 18-27 sequence in 100% of the viral genomes derived from the serum of the other five HLA-A2-positive chronically infected, CTL-nonresponsive, patients was identical to the prototype sequence with the exception of a single conservative substitution of Phe 24 with Tyr in one patient (Table 1). Similarly, the prototype epitope sequence was detected in 2/2 HLA-A2-positive, CTL-responsive patients with acute hepatitis.

In the precore region, base substitutions introducing a stop codon at amino acid positions 28 (G to A at nucleotide 1896) and 29 (G to A at nucleotide 1899) were detected in all viral genomes sequenced from the two chronic patients displaying the variant HBc18-27 epitope (patient 1 and 2 in Table 1) and one acute patient (patient 8 in Table 1). In contrast, the same substitutions were present at both of these positions in 17 and 8% of the viral clones sequenced from chronic patients 3 and 4, respectively, and a single substitution at position 1896 was found in 12% of the DNA clones derived from chronic patient 5. The remaining patients showed prototype precore sequences.

**Table 1.** Sequencing Analysis of the HBc18-27 Region in HLA-A2-positive Patients with Chronic and Acute Self-limited HBV Infection

	Wild type Sequence	HLA-A2 anchor residues										Percent viral genomes
		18 PHE	19 LEU	20 PRO	21 SER	22 ASP	23 PHE	24 PHE	25 PRO	26 SER	27 VAL	
<b>Chronic patients</b>												
1	—	—	—	ASN	—	—	—	—	—	—	ALA	100
2	—	—	—	ALA	—	—	—	—	—	—	ISO	94
	—	—	—	VAL	—	—	—	—	—	—	ISO	6
3	—	—	—	—	—	—	—	—	—	—	—	100
4	—	—	—	—	—	—	—	—	—	—	—	100
5	—	—	—	—	—	—	—	—	—	—	—	100
6	—	—	—	—	—	—	TYR	—	—	—	—	100
7	—	—	—	—	—	—	—	—	—	—	—	100
<b>Acute patients</b>												
8	—	—	—	—	—	—	—	—	—	—	—	100
9	—	—	—	—	—	—	—	—	—	—	—	100

The percentage of DNA clones with wild type or variant sequence is illustrated. The amino acids acting as anchor residues for binding to HLA-A2 are indicated by arrows.

*The Natural Substitutions of HBV Core Residue 27 Reduce the Binding Affinity of Peptide HBc 18-27 for HLA-A2.1.* Peptides containing the amino acid sequence deduced from the natural variants were synthesized to study the influence of the natural substitutions on the binding of the prototype HBc18-27 peptide to HLA-A2.1 molecules. The binding affinity of the variant peptides was compared to that of the prototype 18-27 peptide by testing their capacity to compete with a reference-radiolabeled peptide for the binding to purified HLA-A2.1 molecules, as recently described (36).

As predicted from the crystallographic model of peptide binding to HLA-A2.1 molecules (18), the binding affinity of the peptides carrying amino acid substitutions at position 21 was the same as the prototype peptide (Table 2). In contrast, the binding affinity was decreased 10–20-fold when valine 27 (an anchor residue) was substituted by either isoleucine or alanine (Table 2). Although the binding affinity of the substituted peptides remained within the range of affinity of known HLA-A2-restricted epitopes, it would not be surprising if the decreased affinity might cause the corresponding natural variant viruses to be less immunogenic than the prototype.

*The Natural Substitutions of HBc Residues 21 and 27 Can Abrogate Peptide Recognition by CTL.* A large panel of cytotoxic T cell clones and lines specific for the prototype HBcAg 18-27 sequence was generated from two different HLA-A2-positive patients with acute hepatitis B in order to study the influence of the natural substitutions in the epitope on its ability to be recognized by prototype-specific CTL. The prototype-specific CTL clones were HLA-A2 restricted and were able to recognize endogenous core antigen synthesized within the target cells following infection with recombinant vaccinia virus (29) as efficiently as the synthetic prototype HBc 18-27 peptide (data not shown). Individual CTL clones showed different patterns of reactivity against the variant pep-

tides. More than half (15/29) of the clones tested (e.g., clones M4 and G29 in Fig. 2) were incapable of recognizing peptides containing substitutions at residues 21 and 27 even at very high peptide concentrations. The rest of the clones were less profoundly influenced by the substitutions, requiring higher concentrations of the variant versus the prototype peptide to yield equivalent cytolytic activity (e.g., clones G13, G33, and G3.13 clones in Fig. 2). Similar results were obtained with peptides corresponding to the natural variants from chronic patient 1 (Asn 21 and Ala 27; Fig. 2, top) and chronic patient 2 (Ala or Val 21 and Ile 27; Fig. 2, bottom).

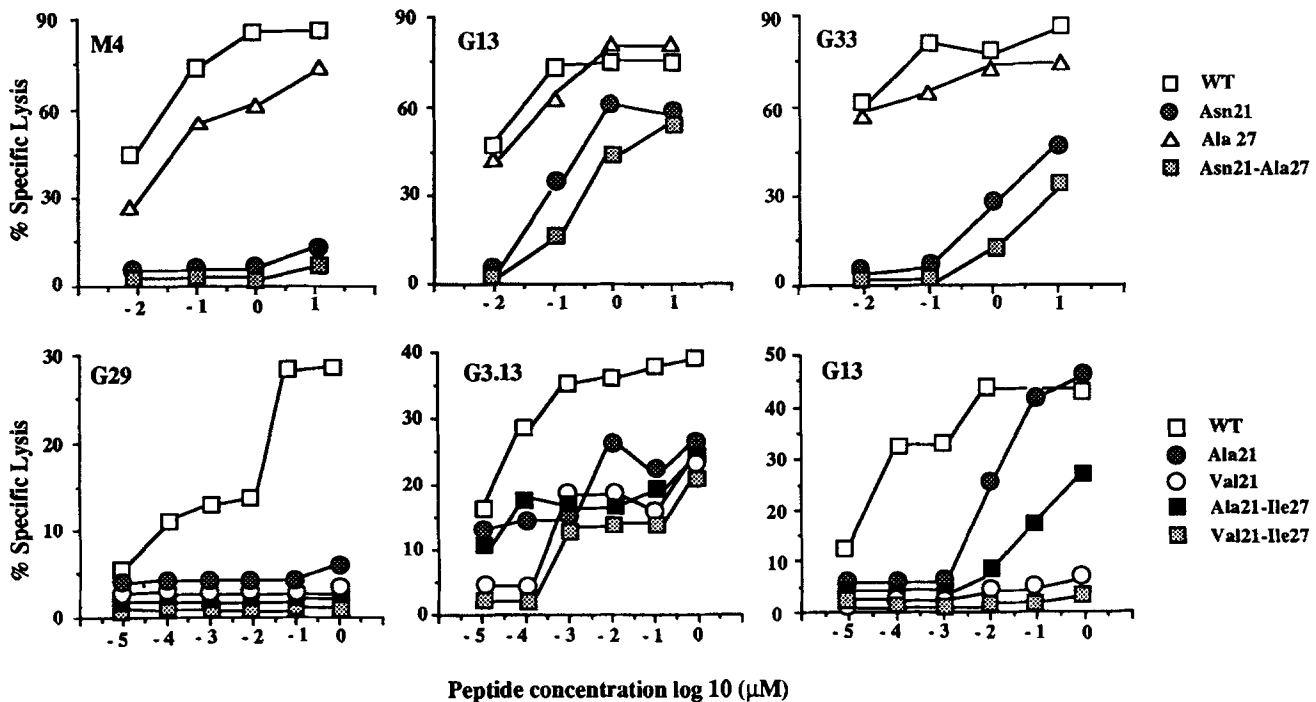
The foregoing results were confirmed with polyclonal CTL lines derived from HLA-A2-positive acute patients by PBMC stimulation with prototype HBc18-27 peptide, indicating that the findings at the clonal level were representative of the polyclonal repertoire within acutely infected patients (Fig. 3, B and C). Furthermore, an oligoclonal line derived from chronic CTL responder patient 1 (infected by the variant HBc Asn 21-Ala 27 virus) by stimulation with the prototype peptide was unable to recognize peptides containing either the double 21/27 substitution or the substitution of residue 21 alone (Fig. 3 A), suggesting that the in vitro observations might be pertinent to in vivo events in the chronically infected patient himself.

Serine at position 21 seemed to be involved in peptide recognition by the TCR because its substitution with either asparagine or alanine or valine abolished peptide recognition (Fig. 2) without affecting HLA binding affinity (Table 2). This was confirmed by competition experiments in which variant Asn 21 or Ala 21 peptides effectively blocked recognition of the prototype peptide by T cell clones (data not shown). In contrast, conservative substitution of the valine anchor residue at position 27 with either alanine or isoleucine, which decreases HLA binding 10–20-fold, (Table 2) imposed a requirement for high peptide concentration for cyto-

**Table 2.** Peptide Binding to Purified HLA-A2.1 Molecules

AA sequence	Binding to HLA-A2.1	
	Fold reduction compared to WT	IC50% nM
FLPSDFFPSV (WT)	—	1.2 ± 0.3
FLPNDFFP SA (Asn21-Ala27)	12.5	15 ± 4.0
FLPNDFFP SV (Asn21)	—	1.4 ± 0.35
FLPSDFFP SA (Ala27)	19	23 ± 0.5
FLPADFFP SI (Ala21-Ile27)	19.2	11 ± 2.4
FLPADFFP SV (Ala21)	—	1.3 ± 0.15
FLPVDFFP SI (Val21-Ile27)	19	23 ± 6.6
FLPVDFFP SV (Val21)	—	1.9 ± 0.35
FLPSDFFP SI (Ile27)	9.1	10.9 ± 3.0

Binding is expressed as IC50% (50% inhibitory concentration) that represents the dose of test competitor peptide yielding 50% inhibition of reference radiolabeled peptide binding.



**Figure 2.** Cytotoxic response of HBc18-27-specific CTL clones against different concentrations of prototype and variant HBc18-27 peptides. CTL clones raised against prototype HBc18-27 were derived from patients with acute self-limited HBV infection. The upper panel shows the CTL response against synthetic peptides corresponding to the variant 21/27 sequence with Asn at position 21 and Ala at position 27 found in chronic patient 1; the lower panel illustrates the results obtained with peptides containing the natural substitutions (Ala or Val 21 and Ile 27) found in chronic patient 2.

lytic activity to be expressed (data not shown). These results strongly suggest that residue 21 is a critical TCR contact site for a large proportion of CTL that recognize the prototype HBc 18-27 sequence, and that substitution of this residue can strongly influence the CTL response by abrogating peptide recognition.

**Induction of the CTL Response by Prototype and Variant HBc18-27 Peptides in HLA-A2-positive Chronic and Acute Patients.** The effect of the naturally occurring substitutions on the induction phase of the cytotoxic response was investigated next, by comparing the capacity of variant and prototype HBc 18-27 peptides to stimulate the CTL response of peripheral blood T cells from HLA-A2 positive, chronic, and acute hepatitis B patients. The length of peptide stimulation was limited to 7 d to minimize the risk of inducing primary *in vitro* CTL responses. As expected, following stimulation of PBMC from chronic patients infected by the variant virus with the prototype peptide, the 21/27 analogue peptide was either recognized less efficiently than the prototype peptide (Fig. 4 A, experiment 1) or not recognized at all (Fig. 4 A, experiments 2 and 3). On the other hand, PBMC stimulation with the variant peptide was ineffective in CTL induction even at peptide concentrations up to 100  $\mu$ M (Fig. 4 B).

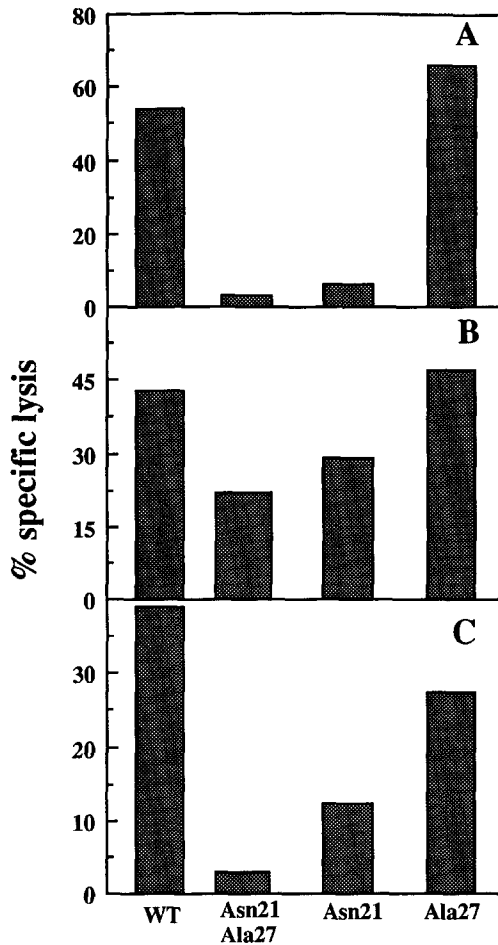
Not surprisingly, CTL induction with 21/27 variant peptides in acute HLA-A2-positive patients infected by viruses that express the prototype peptide was much less effective than induction with prototype HBc 18-27 (Fig. 5 A). Indeed, frequently the variant peptides were totally unable to

expand CTL specific either for themselves or for the prototype sequence, as illustrated by the representative experiment in Fig. 5 B.

It is noteworthy that the single substitution of valine 27 with either alanine or isoleucine completely abolished CTL induction in both acute and chronic patients even at peptide concentrations as high as 1  $\mu$ M that, in contrast, can efficiently sensitize target cells for recognition by 18-27 specific CTL (Fig. 2). Similarly, the substitution of residue 21 with either asparagine or alanine had a drastic negative effect on the induction phase of the CTL response (data not shown).

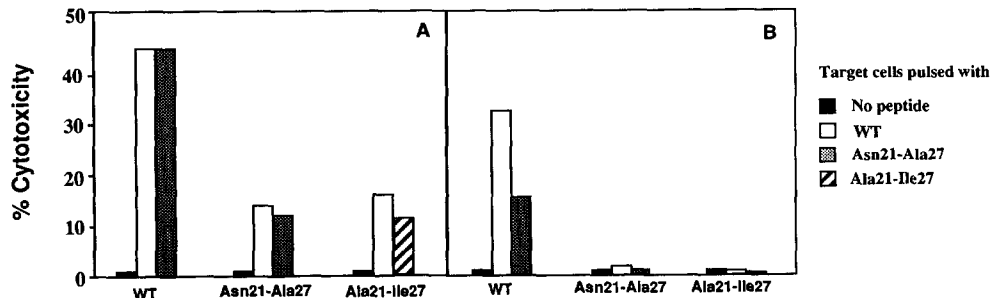
Finally, no cytotoxicity was observed in HLA-A2-positive chronic HBV patients infected by prototype virus and in healthy HLA-A2-positive control individuals following PBMC stimulation with prototype and variant peptides (data not shown). Taken together, these results indicate that the variant HBc 21/27 peptide is less immunogenic than its prototype counterpart, in patients chronically infected by the HBV 21/27 variant and suggest that their *in vivo* CTL responses to the prototype HBc 18-27 sequence are probably ineffective against the prevalent infecting variant virus.

**Analysis of the CTL Repertoire in Patients Chronically Infected by the HBcAg 21/27 Variant and Patients Acutely Infected by the Prototype Virus.** The CTL repertoire in the HLA-A2-positive chronic patient 1 infected by the variant virus was studied using a panel of 77 different peptides, 9- or 10-amino acids in length, selected by scanning the entire amino acid sequence of HBV for the presence of the HLA-A2 binding motif de-

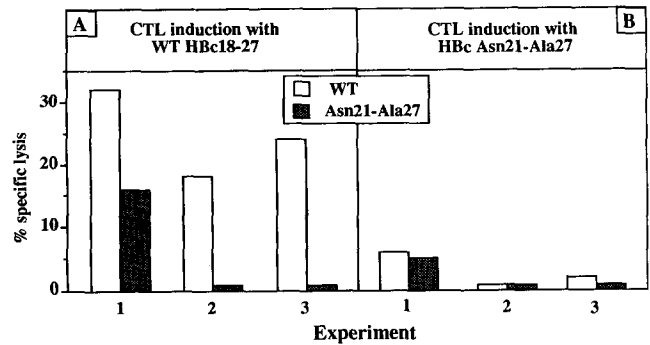


**Figure 3.** Recognition of prototype and variant peptides by an oligoclonal CTL line from chronic patient 1 (A) and two polyclonal CTL lines from patients with acute self-limited hepatitis B (B and C). Production of oligoclonal and polyclonal CTL lines is described in Materials and Methods. Lysis of target cells pulsed with 1  $\mu$ M peptides is illustrated.

scribed by Rammensee et al. (15). Each peptide contained a leucine or isoleucine at position 2 and valine or leucine or isoleucine at the carboxyterminal position. Remarkably, the CTL response in patient 1 chronically infected by the HBc 21/27 variant was selectively focused on the prototype HBc 18-27 sequence (Fig. 6). This is in striking contrast with the multispecificity of the CTL response expressed by patients with acute self-limited hepatitis B, that is simultaneously



**Figure 5.** CTL induction with prototype HBc18-27, variant Asn21-Ala27, and Ala21-Ile27 peptides in two representative patients with acute self-limited HBV infection. Culture conditions as in Fig. 4. In A, prototype and Asn21-Ala27 peptides were recognized with comparable efficiency at high concentrations but the variant 21/27 peptide was recognized less efficiently at low peptide concentrations (not shown).



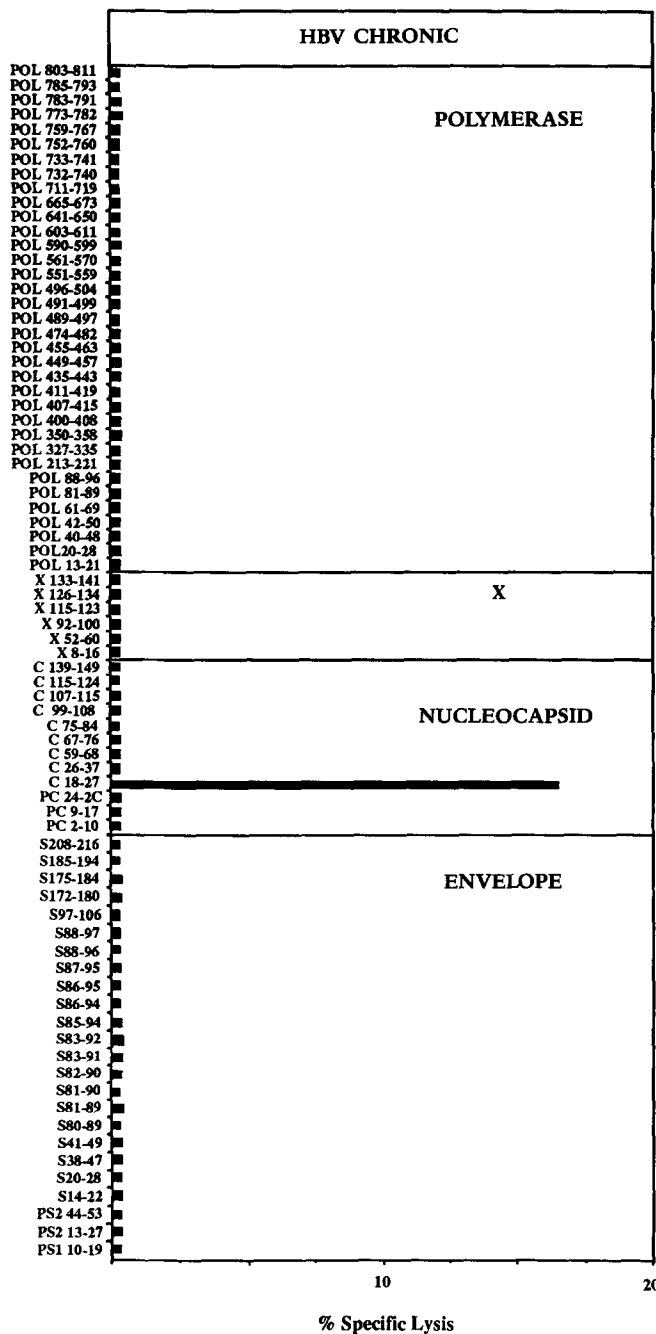
**Figure 4.** CTL induction with prototype HBc18-27 and variant Asn21-Ala27 peptides in chronic patient 1. PBMC were stimulated either with prototype or with variant Asn21-Ala27 peptides (1  $\mu$ M) for 7 d and then tested against HLA-A2-positive target cells pulsed either with prototype or with variant peptides (1  $\mu$ M) or with medium alone. No CTL activity was induced even at concentrations of 10 and 100  $\mu$ M of variant peptide (data not shown).

directed against multiple epitopes scattered throughout the different HBV antigens (26, 30; and data not shown).

### Discussion

Cytotoxic T cells play an essential role in the control of viral infections (38–43). For example, patients with acute HBV infection who successfully clear the virus develop a polyclonal, multispecific CTL response against several epitopes in the viral envelope, nucleocapsid, and polymerase proteins that is vigorous enough to be detectable in the peripheral blood lymphocyte compartment (26, 29–31; and Rehmann, B. et al., manuscript in preparation). In contrast, the peripheral CTL response to HBV is usually undetectable or extremely weak in patients who fail to clear the virus and develop chronic hepatitis (26, 30).

The two patients with chronic active hepatitis B described in the present report represent an exception to this general rule. Despite their persistent infection, for unknown reasons they displayed a strong CTL response against a conserved, HLA-A2-restricted epitope (FLPSDFFPSV) that is located between residues 18-27 of the prototype HBcAg sequence that carries the HLA-A2 binding motif of Rammensee et al. (15). The CTL response to this epitope was comparable in intensity and capacity to recognize endogenously synthesized antigen as the response produced by patients who ul-



**Figure 6.** CTL response against HBV peptides containing the HLA-A2 binding motif. PBMC from chronic patient 1 infected by the Asn21-Ala27 variant virus were stimulated for 7 d with 1  $\mu$ M of each peptide and then tested against HLA-A2-positive target cells pulsed either with the corresponding stimulatory peptide or with medium alone. Identical results were obtained in two further experiments in chronic patient 1 (response focused on Hbc18-27). In two HLA-A2-positive patients with acute self-limited hepatitis B infected by prototype Hbc18-27 virus and studied with the same panel of synthetic peptides, significant levels of cytotoxic activity were expressed simultaneously against several peptides (one within polymerase, three in the nucleocapsid, and two in the envelope region in one patient; one within the x, one in the polymerase, and two in the envelope region in the second patient).

mately clear the virus during an episode of acute viral hepatitis, yet the virus persisted in these patients.

To understand the basis for viral persistence in the presence of this CTL response, we examined the ability of the patients to respond to 79 additional HBV-derived peptides containing the HLA-A2 binding motif, and we determined the DNA sequence of the viruses that infected these patients. Unlike patients with acute hepatitis, the two chronically infected CTL responders failed to respond to any of the other known HLA-A2-restricted CTL epitopes in HBV. Furthermore, they were infected by viruses containing amino acid substitutions of HLA-binding (position 27) and TCR contact (position 21) residues in the prototype Hbc 18-27 epitope that severely impaired its immunogenicity and its ability to be recognized by prototype-specific CTL.

Three important questions emerged from these observations. First, why did these two patients respond at all? Second, why was their response limited to a single epitope? Third, why did the virus persist in the face of this response? While we can only speculate in the most general terms about the influence of host genetics, viral genotype and epitope dominance with respect to the first two questions, the available data permits us to at least discuss two reasonable alternatives for the persistence of a variant virus in the face of a limited CTL response to an epitope that it cannot produce.

Because the identity of the primary infecting viruses cannot be determined by direct analysis, either these two patients were originally infected by the variant genomes, or they were infected by a prototype virus that subsequently mutated and was negatively selected by the prototype Hbc 18-27-specific CTL response that they produced.

For the first hypothesis to be correct the patients would have to be primed *in vivo* by the variant epitope resulting in a CTL response that is neither strong enough to mediate clearance of the variant viruses nor to be boosted by the homologous variant peptides *in vitro*, but is sufficient to be boosted *in vitro* by the prototype epitope (presumably because it displays higher binding affinity than the variant for the HLA-A2 molecule and the CTL T cell receptor). According to this hypothesis, preferential recognition of the prototype Hbc 18-27 epitope might reflect priming *in vivo* by a high concentration of the "low affinity" variant epitope and cross-reactivity with lower concentrations of the "high affinity" prototype sequence. Under these circumstances the CTL could be detectable *in vitro* by their capacity to kill target cells pulsed with low doses of the prototype peptide and high doses of the variant peptides.

The hypothesis is unattractive for several reasons. First, our inability to derive a variant-specific CTL response in either of these two patients, even at very high concentrations of variant peptide (100  $\mu$ M), precludes definitive testing of the idea. Second, since serine 21 in the prototype sequence was shown to be a crucial TCR contact site for polyclonal and oligoclonal T cell lines derived from chronic patient 1 it is most likely that circulating T cell clones that recognize this residue were primed *in vivo* by prototype virus. Third, our own data and the HBV sequence database indicate that the



mutations at positions 21 and 27 of HBcAg are unique features of the viruses infecting the two chronic patients in this study, suggesting that the variants probably originated during the course of the infection in these specific individuals. The finding that substitutions of HBcAg residues 21 and 27 are different in the two chronic patients confirms that they were not infected by a single virus type that might be widespread throughout the geographical area from which they were drawn, and suggests that these variants may be relevant only to the individuals within which they evolved.

Several observations make the second hypothesis more attractive: first, the CTL repertoire is uncommonly narrow in the patients infected by the variant virus and apparently focused on the HBV region where mutations arose; second, natural substitutions can abrogate the cytotoxic activity of CTL specific for the prototype epitope; third, the variant peptide is poorly immunogenic compared to the prototype. However, this hypothesis remains indirect because we did not observe a conversion from prototype to variant viral genotype in these patients. Additionally, it requires an explanation for the paradoxical persistence of a strong, sustained CTL response to the prototype epitope in the absence of detectable prototype virus. The paradox can be resolved if one postulates either recurrent stimulation by prototype peptide periodically presented to CTL recognition from a currently undefined reservoir of prototype virus or very long-term memory of a previous encounter with prototype virus (44–48). Consistent with the first possibility, it has recently been demonstrated (49) that intravascular HBV-specific, class I-restricted CTL that can recognize and destroy HBV antigen-positive hepatocytes in transgenic mice are simultaneously unable to recognize the same antigens expressed in other tissues (e.g., brain, kidney, testis, pancreas, gastrointestinal tract, etc.) in the same animals. Further, it has been shown that the CTL ignore extrahepatic viral antigen because their access to antigen at these sites is precluded by microvascular anatomical barriers in these tissues that do not exist in the sinusoidal structure of the liver. Based on these observations, it has been suggested that similar processes may contribute to viral persistence during natural infections. In view of these findings, it is conceivable that these patients may have been infected

by a prototype virus that was eliminated from the liver (but not from these putative inaccessible extrahepatic sites) by the CTL response to a single prototype epitope which subsequently led to negative selection of the variant genome that eventually dominated the intrahepatic virus population. According to this scenario, the HBV prototype would persist in the extrahepatic tissues despite a vigorous prototype-specific CTL response in the periphery, and prototype virions released from such protected sites could infect liver cells where they can maintain the prototype-specific CTL response as well as the associated chronic liver disease.

Although we favor the foregoing scenario to explain the results of this study, it is important to remember that 100% of the viral genomes in these two patients contained substitutions at nucleotides 1896 and 1899 that introduce a stop codon in the precore region. It is not likely that this property could have led to immune selection of the HBc18-27-variant viruses described in this report. However, if this or other associated mutations were eventually proven to confer a growth advantage to the variant virus, then selection could be explained on this basis, rather than by the pressure exerted by the CTL response.

In conclusion, we believe that our results are most compatible with the concept that variant viruses carrying substitutions within an immunodominant CTL epitope were selected by CTL pressure in these two patients because the anti-viral CTL response was narrowly focused on the prototype epitope and because the variant epitopes did not induce a new population of CTL specific for themselves. It is important to emphasize that while the results of our study may serve to demonstrate that such host-virus interactions can and do occur during naturally acquired HBV infection in man, we do not suggest that this process is responsible for viral persistence in most patients with chronic HBV infection. Even if negative selection of CTL escape mutants eventually proves to be common during chronic HBV infection, their occurrence is likely to accompany and aggravate, not cause, viral persistence which is likely due primarily to the development of a weak class I and II-restricted cellular immune response to the known viral antigens (26, 30, 50).

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*Note Added in Proof:* Experiments completed when the manuscript was already submitted demonstrate that the natural substitutions of residues 21 and 27 of HbcAg can interfere with the recognition of the prototype epitope Hbc18-27 also by a mechanism of TCR antagonism (Bertoletti, A., A. Sette, F. V. Chisari, A. Penna, M. Leverero, M. De Carli, F. Fiaccadori, and C. Ferrari. 1994. Natural variants of cytotoxic epitopes are T cell receptor antagonists for anti-viral cytotoxic T cells. *Nature (Lond.)* 369:407).

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