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HLA class I-restricted human cytotoxic T cells recognize endogenously synthesized hepatitis B virus nucleocapsid antigen

(viral pathogenesis/viral immunology/synthetic peptides/eukaryotic expression vector/acute hepatitis)

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ABSTRACT **Knowledge of the immune effector mechanisms responsible for clearance of hepatitis B virus (HBV)-infected cells has been severely limited by the absence of reproducible systems to selectively expand and to characterize HBV-specific cytotoxic T lymphocytes (CTLs) in the peripheral blood of patients with viral hepatitis. By using a strategy involving sequential stimulation with HBV nucleocapsid synthetic peptides followed by autologous, or HLA class I-matched, HBV nucleocapsid transfectants, we now report the existence of CTLs able to lyse target cells that express endogenously synthesized HBV nucleocapsid antigen in the peripheral blood of patients with acute viral hepatitis B. The CTL response is HLA-A2 restricted, mediated by CD8-positive T cells, and specific for a single epitope, located between amino acid residues 11 and 27 of HBV core protein; these residues are shared with the secretable precore-derived hepatitis B e antigen. Equivalent lysis of target cells that express each of these proteins suggests that their intracellular trafficking pathways may intersect. The current report provides definitive evidence that HLA class I-restricted, CD8-positive CTLs that recognize endogenously synthesized HBV nucleocapsid antigen are induced during acute HBV infection in humans and establishes a strategy that should permit a detailed analysis of the role played by HBV-specific CTLs in the immunopathogenesis of viral hepatitis.**

The hepatitis B virus (HBV) is an enveloped, circular, double-stranded DNA virus that infects hepatocytes and causes acute and chronic liver disease and hepatocellular carcinoma (1). The pathogenetic mechanisms responsible for liver cell injury in HBV infection are not well understood, although it appears that the virus is not directly cytopathic for the infected hepatocyte. Based on correlative clinical observations and precedent from other viral systems (2, 3), it is thought that liver cell injury is mediated by an HLA class I-restricted, CD8-positive cytotoxic T-lymphocyte (CTL) response to one or more HBV-encoded antigens (4).

Since HBV does not readily infect human cells *in vitro*, the experimental approach to this question has been limited. The most relevant data thus far have been produced by Mondelli *et al.* (5, 6); they showed that peripheral blood lymphocytes from patients with acute and chronic hepatitis B may kill autologous hepatocytes *in vitro*. Due to technical limitations inherent in those early studies, the precise specificity of the cytolytic activity, its HLA restriction elements, and its cellular phenotype could not be definitively established; and only patients undergoing diagnostic liver biopsies could be studied.

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More recently, the presence of HBV envelope and core-specific CD8-positive T cells within the liver of patients with chronic active hepatitis B has been reported (7, 8), but their capacity to display cytolytic activity against target cells expressing endogenously synthesized HBV antigens could not be investigated because of the absence of reliable target systems for this analysis.

In the current study we define an experimental strategy, employing sequential stimulation of peripheral blood lymphomononuclear cells (PBLs) with synthetic HBV peptides and autologous transfectants that express the corresponding HBV antigens, that permits the reproducible identification and characterization of low-frequency, HBV-specific CTLs in the peripheral blood of patients with viral hepatitis. This approach provides the basis for longitudinal analysis of the cytotoxic T-cell response during HBV infection and for a definitive clarification of its pathogenetic role in HBV-related liver disease.

MATERIALS AND METHODS

Patients. Three male patients (J.P., V.J., and M.B.) with acute hepatitis B were studied.

Diagnosis of acute hepatitis was based on the finding of elevated values of serum glutamic-pyruvic transaminase (SGPT) activity (at least 10 times the upper level of the normal; mean SGPT peak value = 2179 units/liter), associated with the detection of IgM antibodies directed against the HBV core protein in the serum. All patients recovered completely from the illness, with normalization of serum transaminase and clearance of HBV surface antigens. They were antibody negative to the δ antigen and to the hepatitis C virus.

HLA typing was as follows: patient J.P., A2 A3 B44 B35 Cw4 DR1 DR2 DRw8 DQw1; V.J., A2 A11 B44 B62 Cw5 DR4 DRw12; M.B., A2 B38 B27 DR5 DRw52 DQw3.

Expression Vectors. Two different eukaryotic expression systems were employed in these studies. The HBV envelope and core open reading frames were inserted into the Epstein–Barr virus (EBV)-based plasmid vector EBO-pLPP (9), which is maintained as an episome when transfected into EBV-transformed human B-cell lines (BCLs). The HBV inserts are under the transcriptional control of the simian virus 40 early promoter and polyadenylylation signal. The EBO-transfected cells were selected with hygromycin (250 μg/ml; Sigma), a selection marker carried by the plasmid.

Abbreviations: HBV, hepatitis B virus; CTL, cytotoxic T lymphocyte; PBL, peripheral blood lymphomononuclear cell; EBV, Epstein–Barr virus; HBcAg, hepatitis B core antigen; rHBcAg, recombinant HBcAg; BCL, B-lymphoblastoid cell line; HBc-(11–27), peptide representing amino acids 11–27 of the HBV core protein.

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Plasmids EBO-core and EBO-env encode the HBV core and envelope polypeptides (subtype ayw), respectively. Stable expression of HBV antigens has been obtained following electroporation of EBV-transformed B cells in the presence of each one of the plasmids carrying a specific HBV DNA sequence. The construction and characterization of these vectors will be reported elsewhere (S.G., R.M., P.F., G. Portillo, and F.V.C., unpublished results).

Additionally, recombinant vaccinia viruses that express either the HBV core (Vcore) or precore (Vprecore) polypeptides (subtype ayw) were constructed and used as described (10).

Synthetic Peptides and HBV Antigens. The complete panel of synthetic peptides, 10–20 residues long, corresponding to the complete sequence of the core and precore region-encoded polypeptides (subtype ayw) were kindly provided by Cytel (La Jolla, CA) or purchased from Multiple Peptide Systems (La Jolla, CA). Recombinant hepatitis B core antigen (rHBcAg) was obtained from bacterial extracts of *Escherichia coli* as described (11).

Production of Polyclonal CTL Lines. PBLs were isolated by Ficoll/Hypaque density gradient centrifugation and cultured at 4×10^6 cells per ml in RPMI 1640 containing 10% AB serum plus either a pool of five synthetic core peptides including the peptide representing amino acids 11–27 of the HBV core protein [HBc-(11–27)] (each peptide at 10 μg/ml) or the HBc-(11–27) peptide (10 μg/ml) and rHBcAg (1 μg/ml; Biogen) in a 24-well plate (Corning). Recombinant interleukin 2 (20 units/ml, Hoffmann–La Roche) was added after 4 days of culture. The peptide-primed cells were restimulated weekly following three different strategies: (*i*) stimulation with HBc-(11–27) peptide and rHBcAg in the presence of autologous irradiated (3500 R) PBLs as antigen-presenting cells (5×10^5/ml; patient V.J.), (*ii*) stimulation with phytohemagglutinin at 1 μg/ml in the presence of allogeneic irradiated (7000 R) PBLs (5×10^5/ml; patient J.P.), (*iii*) stimulation with irradiated (7000 R) HLA class I-matched EBO-core transfectants (1×10^5/ml) plus autologous irradiated (3000 R) PBLs (5×10^5/ml) and rHBcAg (patient V.J.).

Cytotoxicity Assay. Cytotoxic activity was assessed by incubating the peptide-primed effector T-cell lines with autologous or allogeneic (HLA-matched or -mismatched) ^{51}Cr-labeled BCLs for 4 hr in round-bottomed 96-well plates. Target BCLs were either incubated with synthetic peptides (20 μg/ml for 1 hr); infected with recombinant vaccinia viruses (1 hr of infection followed by 13 hr of expression, using a multiplicity of infection of 20); or stably transfected with EBO plasmid vectors. The percent target cell lysis was calculated from the formula $[(E - M)/(D - M)] \times 100$, where E = experimental ^{51}Cr release (cpm); M = ^{51}Cr release in presence of culture medium (which ranged between 15% and 25% of total releasable counts); D = total ^{51}Cr released by 10% Triton X-100.

In inhibition experiments, CTL lines were tested against HLA-A2-positive EBO-core- and EBO-env-transfected BCL targets in the presence or absence of saturating concentrations of the IgG1 monoclonal antibodies anti-Leu-3a (CD4) and anti-Leu2a (CD8), purchased from Becton Dickinson. Antibodies (0.6 μg/ml) were added to the culture at the initiation of the chromium-release assay.

RESULTS AND DISCUSSION

Three HLA-A2-positive patients (M.B., J.P. and V.J.) were studied during the acute phase of viral hepatitis type B. PBLs from these patients were analyzed for HBV-specific CTL activity either (*i*) immediately after isolation, (*ii*) after 1 or 2 weeks of stimulation with autologous stimulator cells transfected or infected with the HBV expression vectors, or (*iii*) after stimulation with a panel of four pools of overlapping synthetic peptides each one consisting of five or six peptides covering the entire HBV nucleocapsid region (ayw subtype).

HBV-specific CTL activity was reproducibly observed only following stimulation with one of the four peptide pools, and recognition was limited to a single peptide consisting of residues 11–27 of the hepatitis B core protein [HBc-(11–27); data not shown]. The single-letter code amino acid sequence of this peptide is ATVELLSFLPSDFFPSV, which is conserved among the major subtypes of HBV. As will be reported elsewhere, this is a dominant epitope in HLA-A2-positive patients with acute viral hepatitis (26).

After 1 week of stimulation with a peptide mixture containing the HBc-(11–27) determinant, lymphocytes from patient M.B. specifically lysed autologous and HLA-A2-matched BCLs incubated with the peptide (Fig. 1*A*). A peptide-specific CTL line was also established from PBLs derived from patient V.J. (Fig. 1*B*) by stimulation with the HBc-(11–27) plus rHBcAg, as a source of antigen-specific T-cell help (12), followed by restimulation with the same

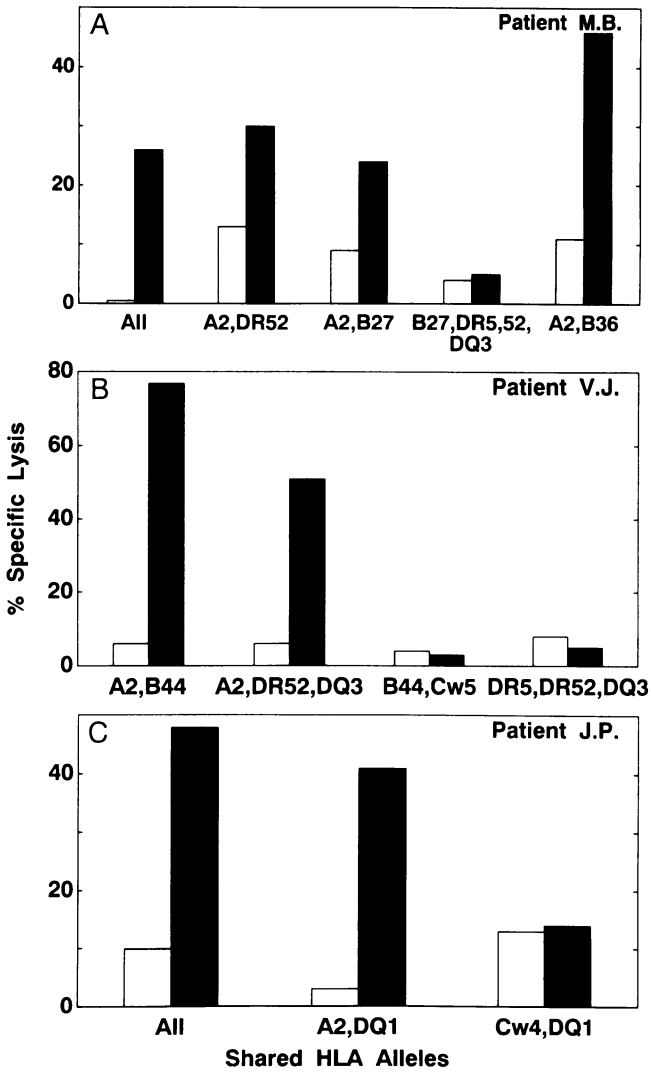

FIG. 1. Identification of HBc-(11–27) peptide-specific, HLA-A2-restricted CTLs in acute viral hepatitis. HBc-(11–27) peptide-specific CTLs were generated from PBLs from three patients with acute hepatitis B (M.B., J.P., and V.J.) as described. The effector-to-target ratios were 100 (M.B.) or 10 (V.J. and J.P.). Cytotoxic activity was assessed after 7 (M.B.) or 14 (V.J. and J.P.) days of culture. The HLA alleles shared between each patient and target cells are shown. Before ^{51}Cr-labeling, target cells (EBV-transformed BCLs) were pulsed with the HBc-(11–27) peptide (solid bars) as described. BCLs not pulsed with peptide served as negative controls (open bars).

reagents. Similarly, an HBc-(11–27)-specific CTL line was established by using PBLs from patient J.P. (Fig. 1*C*) by 1 week of peptide and HBcAg stimulation followed by restimulation with phytohemagglutinin. All strategies resulted in establishment of short-term CTL lines that were able to lyse peptide-pulsed autologous and allogeneic HLA-A2-positive target cells but not HLA-A2-mismatched targets, demonstrating that peptide recognition by the CTLs was HLA-A2 restricted (Fig. 1).

The short-term, peptide-specific CTL lines from all three patients were tested for their capacity to recognize endogenously synthesized core antigen using autologous and allogeneic BCL targets transfected or infected with the HBV core expression vectors. As shown in Figs. 2 and 3, such CTLs could lyse only autologous BCLs expressing the HBV core polypeptide. By using a panel of HLA-A2-matched and -mismatched allogeneic target cells, the specific recognition was also shown to be HLA-A2 restricted (Figs. 2 and 3*C*).

However, stimulation with peptides can in some instances induce CTLs with low affinity for the corresponding endogenous protein (13), such that stimulation may yield CTLs that recognize peptide but not native antigen. When this phenomenon takes place, selective expansion of the CTL populations that recognize native antigen can be induced by repetitive stimulation with endogenously synthesized antigen (13). Following this approach, PBLs from patient V.J. were stimulated for 2 weeks with peptide HBc-(11–27) plus rHBcAg, after which the PBLs were restimulated with HLA-matched transfected BCLs that stably express the HBV nucleocapsid polypeptide.

Prior to restimulation, the CTLs displayed a high level of cytotoxicity toward peptide-pulsed targets with only minimal specific killing of target cells expressing endogenously synthesized antigen (Fig. 3*A*). After restimulation with the nucleocapsid transfectants, T cells displayed increased killing of targets that express endogenously synthesized antigen concomitant with a decrease in killing of peptide-pulsed target cells (Fig. 3*A*). Indeed, recognition of endogenously synthesized antigen increased progressively with time following restimulation (Fig. 3*B*). The foregoing results suggest either that naturally processed nucleocapsid antigen is similar, but not identical, to the synthetic HBc-(11–27) peptide used to expand the CTL precursor population or that the natural and the synthetic peptides can bind in a different way to the same HLA molecule (i.e., different orientation within the HLA groove), thereby leading to the selection of T-cell

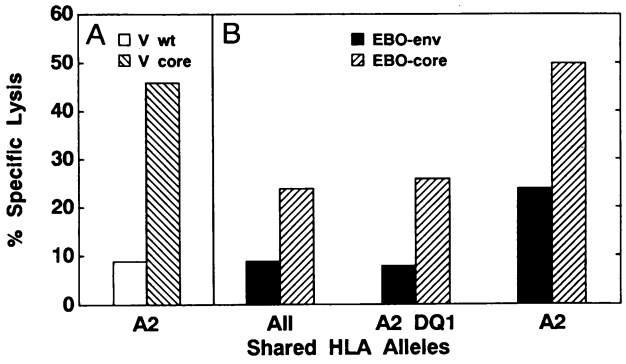

FIG. 2. Recognition of endogenously synthesized HBV nucleocapsid antigen by peptide-specific CTLs. Effector cells derived from patients M.B. (*A*) and J.P. (*B*) after 7 and 14 days, respectively, of stimulation with the HBc-(11–27) peptide were incubated with target cells for 4 hr at effector-to-target ratios of 100:1 and 10:1, respectively. Before ^{51}Cr-labeling, BCL targets were either infected with recombinant vaccinia viruses (V wt = wild-type vaccinia; V core = vaccinia carrying the HBcAg open reading frame; *A*) or transfected with EBV-based episomal vectors (EBO-env and EBO-core; *B*).

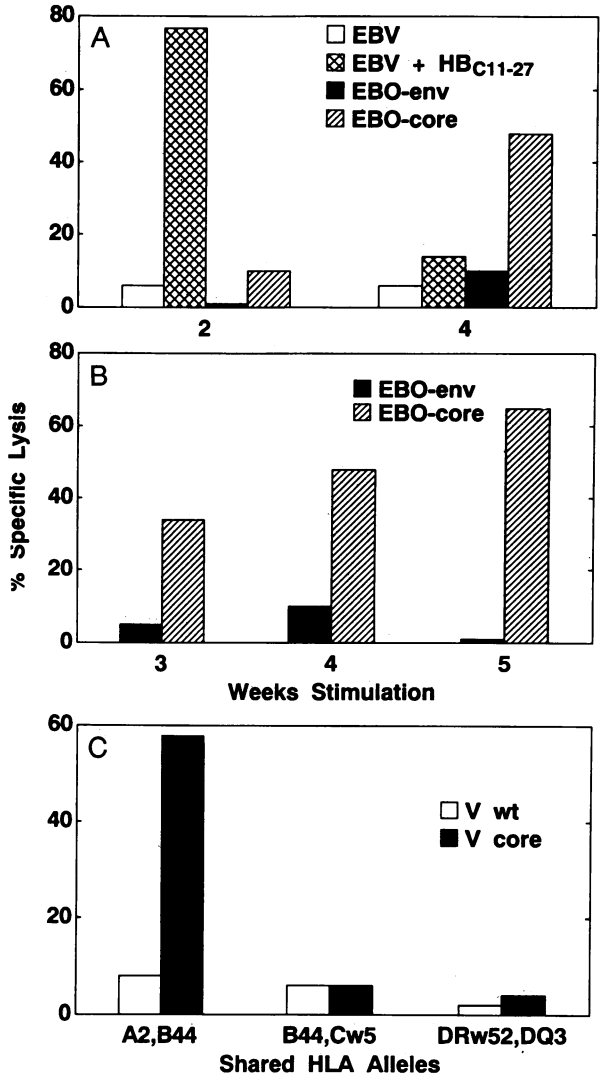

FIG. 3. Selective expansion of HLA-A2-restricted CTLs that recognize endogenously synthesized HBV nucleocapsid antigen. After 2 weeks of stimulation with HBc-(11–27) peptide and rHBcAg, the peptide-specific CTL line V.J. (Fig. 1*B*) was further restimulated every 7 days with irradiated HLA class I-matched EBO-core transfectants (see *Materials and Methods*). (*A*) The cytolytic activity was tested against HLA-A2-matched BCLs either prepulsed with HBc-(11–27) peptide or cultured in medium alone and against EBO transfectants expressing endogenously synthesized nucleocapsid or envelope antigens. Experiments were performed before (2 weeks) and after (4 weeks) two rounds of stimulation with EBO-core transfectants (effector-to-target cell ratio = 20:1). (*B*) Recognition of endogenously synthesized HBV nucleocapsid antigens by the CTL line V.J. during weeks 3, 4, and 5 of culture after 1, 2, or 3 sequential rounds of stimulation with EBO-core transfectants (effector-to-target cell ratio = 20:1). (*C*) Cytolytic activity toward HLA-A2-matched and -mismatched BCL targets infected with recombinant vaccinia viruses (designated as in Fig. 2) after 5 weeks of culture (effector-to-target cell ratio = 50:1).

receptors with different fine specificity. Importantly, other investigators (14–16) have recently demonstrated that naturally processed antigen is present in the HLA class I binding pocket as an 8 or 9 amino acid residue fragment, which is considerably shorter than the HBc-(11–27) peptide we used in this study.

The fact that this sequential stimulation strategy worked so well, when many prior attempts to detect HBV-specific CTLs in freshly isolated PBL without prior stimulation failed, suggests that the HBV-specific CTL precursors are probably

present in the peripheral blood compartment at very low frequency. If this assumption is correct, the current strategy may be generally useful for the detection of low-frequency, HLA class I-restricted CTLs in other systems where the antigenic specificity is known. Our failure to induce HBV-specific CTLs solely by *in vitro* stimulation with stably transfected autologous BCLs that, nonetheless, serve as excellent target cells is compatible with the recent demonstration (17) that much higher epitope densities are required for CTL induction compared with the density required for lysis. Once again, the current strategy would serve to circumvent this limitation in other systems in which the level of endogenous expression of the relevant antigen is below the threshold needed to selectively expand the CTL *in vitro*.

The phenotype of the HBc-(11–27)-specific CTL was assessed by incubating HBV core transfectants and the nucleocapsid-specific CTL line from patient V.J. with antibodies specific for the differentiation markers CD4 and CD8. Antigen-specific lysis was blocked by 80% with antibodies to CD8, whereas no inhibition was obtained with antibodies to CD4 (data not shown). As expected, these results demonstrated that the HBc-(11–27)-specific, HLA-A2-restricted CTL activity was mediated exclusively by CD8-positive T cells.

The HBc-(11–27) epitope is located within two independent nucleocapsid polypeptides (core and precore), one of which (precore) contains an amino-terminal signal sequence that leads to its translocation into the endoplasmic reticulum and secretion as hepatitis B e antigen (HBeAg) (18–20), whereas the other (core) is primarily a cytoplasmic and nuclear protein (HBcAg) and is not secreted (21, 22). To determine whether one or both polypeptides serve to generate the HLA-A2-restricted HBc-(11–27) CTL epitope, BCL target cells were infected with recombinant vaccinia viruses engineered to express the native core and precore polypeptides independently (10). Both target cell lines were killed to an equivalent degree by an HBc-(11–27)-specific CTL line from patient J.V. (Fig. 4), demonstrating that HBcAg and hepatitis B e antigen share a common intracellular processing pathway and that they are crossreactive at the HLA class I-restricted CTL level, as previously shown for class II-restricted helper T cells (23, 24).

In previous experiments we have demonstrated that an HLA class I-restricted, HBV envelope-specific CTL response can be induced in inbred mice by infection with a

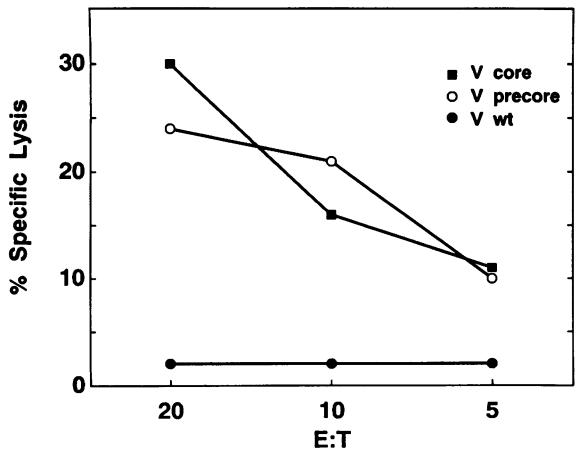

FIG. 4. HBc-(11–27)-specific CTLs recognize an epitope shared by the HBV core and precore region-encoded polypeptides. CTL line V.J. was tested against HLA-A2-positive BCLs infected with recombinant vaccinia viruses expressing either the HBV core (V core) or precore (V precore) polypeptides at the indicated effector-to-target (E/T) ratios. V wt, wild-type vaccinia virus.

Table 1. Recognition of the HBc-(11–27) sequence by peptide-primed PBLs from uninfected healthy subjects and patients with acute viral hepatitis B

Subject	% specific lysis	
	Medium	HBc-(11–27)
Uninfected control		
1	4	4
2	0	0
3	6	0
4	0	0
5	1	0
6	4	2
7	2	4
8	0	0
Infected patient		
1	0	39
2	0	37
3	0	47

BCLs from HLA-A2-positive uninfected healthy subjects and from HLA-A2-positive patients acutely infected with HBV were prepulsed with medium or with HBc-(11–27) and used as targets in a 4-hr ^{51}Cr release assay as described in the legend to Fig. 1. PBLs were primed with peptide HBc-(11–27) for 1 week prior to analysis.

recombinant vaccinia virus and that specific CTL clones can induce liver cell injury following adoptive transfer into syngeneic transgenic mice that express the corresponding antigen in the liver (25). Although these experiments clearly demonstrate that the hepatocyte has the capacity to process and present an HBV-encoded antigen to a productively rearranged specific CD8-positive T-cell receptor and to be killed in the process, they did not establish the immunogenicity of HBV with respect to the induction of a CTL response during natural infection, since the mouse is not susceptible to infection by HBV. The current report establishes this fact, and to our knowledge it represents the first definitive demonstration that HBV nucleocapsid-specific CTLs are induced *in vivo* during HBV infection in humans.

Since these CTLs recognize endogenously synthesized antigen, they have the potential capacity to play an important role in the pathogenesis of viral clearance and liver cell injury. Importantly, this CTL response was undetectable in uninfected healthy normal controls (Table 1) and significantly weaker or completely absent in patients with chronic HBV infection (26), indicating that it is not merely induced *in vitro* and that, like the HBcAg-specific helper T-cell response (12), it is specific for the acute phase of HBV infection and probably associated with the clearance of infected hepatocytes.

The current system will permit a detailed analysis of many aspects of the human HBV-specific CTL response that have heretofore not been approachable. It will be interesting to see if other HBV proteins serve as CTL targets in acute hepatitis and/or during persistent viral infection. It will also be possible to determine if the clinical course of acute disease, especially its progression to chronicity, is related to the magnitude of the HBV-specific CTL response, to the epitope selected, or to the restriction element by which it is presented to the T cell. Based on these studies, it may eventually be possible to design specific immunotherapeutic strategies to reduce the severity of liver cell injury in patients with chronic hepatitis and thereby to reduce the attendant risk of hepatocellular carcinoma.

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