Purification and Characterization of a Naturally Processed Hepatitis B Virus Peptide Recognized by CD8$^+$ Cytotoxic T Lymphocytes

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Abstract

In vitro studies in patients with hepatitis B virus (HBV) infection have suggested that hepatocytolysis induced by CD8$^+$ cytotoxic T lymphocytes (CTLs) is the most important effector pathway in eliminating infected cells. The recognition is implicated in the endogenously processed HBV antigens in the context of HLA class I molecules presented on the liver cell membrane. However, the naturally occurring HBV peptide antigens have not yet been demonstrated. We report here that a naturally processed peptide antigen P2 was isolated from HLA class I molecules of HBV-infected liver cell membrane. The P2 peptide exhibited the activity of sensitizing target cells for lysis by CD8$^+$ CTLs. The P2 sequence (YVNVNMGLK) purified from liver tissue was in concordance with that encoded by the viral genome for the HBV nucleocapsid antigen or HBcAg 88–96. P2 peptide could also be isolated from the EBV-transformed B cells that were transfected by HBcAg-expressing vector. The P2 epitope, sharing the HLA-A11 binding motifs, was recognized by HLA-A11–restricted CD8$^+$ CTLs. The data provided direct evidence that, in hepatitis B patients, antigenic peptides of HBV were processed by hepatocytes, presented with the class I MHC molecules, and recognized by CD8$^+$ CTLs. (*J. Clin. Invest.* 1996. 97:577–584.) Key words: hepatitis B virus • hepatocytolysis • mutation clustering region • naturally occurring peptide • T cell epitope

Introduction

It is estimated that more than 250 million people throughout the world are chronically infected with the hepatitis B virus (HBV)[1], which is the primary cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma in the endemic areas (1, 2). The mechanisms responsible for the variable clinical course and outcome of HBV infection are not known. There is considerable evidence suggesting that HBV is not directly cytopathic (3), and that the hepatitis is caused by immune responses against virus-infected hepatocytes (4). In vitro studies have shown that hepatitis B surface antigen (HBsAg)– and hepatitis B core antigen (HBcAg)–expressing transfectants can serve as targets for MHC class I–restricted cytotoxic T lymphocytes (CTLs) that are generated from both acute and chronic hepatitis B patients (5–12). In addition, expression of HBcAg in the cytoplasm and on the membrane of liver cells is positively correlated with the hepatitis activity (13, 14). Also, acute exacerbations of chronic type B hepatitis are accompanied by increased T cell responses to HBcAg and hepatitis B e antigen (HBeAg), but not to HBsAg (15). Likewise, we have shown that the expression of HLA class I antigens on hepatocyte membrane is enhanced in association with the development of hepatitis activity in chronic HBV infection (16). These findings suggest that antigenic peptides derived from HBcAg or HBeAg in the context of HLA class I antigens may be important targets for immune-mediated hepatocytolysis in HBV infection. However, it has not yet been demonstrated whether naturally processed HBV peptides recognizable by CTL are presented on the liver cell membrane. To extend our previous observations (14–16), we eluted the naturally occurring peptides from HBV-infected liver tissue and defined the CD8$^+$ T cell epitopes by CTL activities. We demonstrated here one of the identified epitopes capable of sensitizing HLA-A11–positive target cells to lysis by CD8$^+$ CTL lines.

Methods

The strategy used in this study was according to previous reports (reviewed in 17–19) with some modifications (Fig. 1).

Liver tissue

One male patient (HLA-A11; -B57(17), -B60(40); -Cw3) who was seropositive for HBsAg and HBV DNA was diagnosed as having hepatocellular carcinoma with elevated serum alanine aminotransferase levels. A tumor measuring ~ 5 cm in diameter at lateral segment of right lobe was resected. The fresh nontumor part of the removed liver tissue was isolated, and ~ 40 g of liver tissue was immediately perfused with ice-cold 0.154 M NaCl solution for elution of naturally occurring peptides. In addition, a small piece of fresh nontumor tissue was immersed in culture medium to obtain the infiltrating mononuclear cells in liver and to perform T cell cloning by limiting dilution method.

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1. *Abbreviations used in this paper:* β_2M, β_2-microglobulin; CTL, cytotoxic T lymphocyte; ESI-MS, electrospray ionization mass spectrometry; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; MCR, mutation clustering region; TCC, T cell clone; TFA, trifluoroacetic acid.

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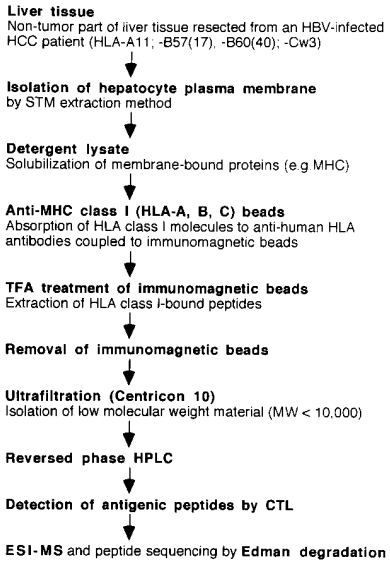

Figure 1. Flow chart shows the strategy of extraction and characterization of naturally occurring peptides from liver tissue. *HCC,* hepatocellular carcinoma; *STM,* 0.25 M sucrose, 5 mM Tris-HCl, pH 7.2, 1 mM $MgCl_2$, d = 1.03 g/ml. For details see Methods.

Isolation of hepatocyte plasma membrane

No attempt was made to isolate the complete cellular peptide pool from the entire hepatocyte, because it needed tremendous work in further purification and screening. We separated hepatocyte plasma membranes for subsequent peptide purification by a standard sucrose, Tris-HCl, and $MgCl_2$ extraction method (20). This is based on the notion that $CD8^+$ CTLs recognize membrane-bound, MHC class I–presented peptide antigens.

Solubilization of membrane-bound proteins

The plasma membrane fraction was solubilized in 0.5% NP-40, 20 mM Tris-HCl, pH 8.0, and 1 mM PMSF (all from Sigma Chemical Co., St. Louis, MO) and then incubated for 60 min to extract both integral membrane proteins and peripheral membrane proteins (21). Insoluble membranes were removed by centrifugation at 100,000 *g* for 60 min.

Affinity purification of HLA class I molecules and acid extraction of HLA class I–bound peptides

The solubilized membrane fraction was mixed with immunomagnetic beads coated with anti–human MHC class I (HLA-A, B, C) antibodies (Dynabeads® HLA class I; Dynal Inc., Great Neck, NY) at 4°C overnight according to the manufacturer's instructions. Subsequently, the beads were collected from suspension by a magnet (Dynal Inc.) and subjected to acid extraction by adding 0.1% trifluoroacetic acid (TFA). The acid extract was lyophilized and redissolved in 0.7% TFA for ultrafiltration through a Centricon 10 membrane (molecular mass cutoff 10 kD; Amicon, Inc., Beverly, MA) to separate bound peptides.

HPLC fractionation of peptides

Low–molecular weight material (mol wt < 10,000) collected from ultrafiltration was loaded onto a C18 reverse-phase column (218TP54; VYDAC, Hesperia, CA). HPLC was performed (Beckman System Gold equipment; Beckman Instruments, Inc., Fullerton, CA) with absorbance monitored at 220 nm. Gradients were generated by using an increasing concentration of acetonitrile from 0 to 30% in 0.1% TFA within 60 min. Fractions (1.0 ml) collected at 1-min intervals were lyophilized and redissolved in distilled water for further analysis. The T cell function assay active fractions were refractionated on HPLC by a slightly modified gradient system (15–30% acetonitrile in 0.1% TFA within 12.5 min; fraction collection/0.5 min).

Detection of antigenic peptides by CTL

The detection requires effector cells (i.e., CTLs), target cells, and the cytotoxicity assay system.

Target cells. EBV-transformed B cell line (EBV-B cell) was generated from PBMC of the patient studied. Subsequently, his EBV-B cells were transfected with the Simian virus 40–derived plasmid pSV2A-Neo-$(HBV)_2$ and the cytomegalovirus-derived plasmid pCMV-core (kind gifts of Dr. J.-H. Ou, University of Southern California, Los Angeles, CA) and selected with the neomycin analogue G418 by the procedures described previously (22, 23). EBV-B cells transfected with pSV2A-Neo-$(HBV)_2$ that could express all the HBV proteins, including HBsAg, HBeAg, and HBcAg, were designated as HBV-transfected cells, and those transfected with pCMV-core capable of expressing HBcAg were referred to as HBc-transfected cells. Nontransfected EBV-B cells from different HLA class I haplotypes, including HB1 (HLA-A11, -A33; -B51, -B55; -Cw1), HB2 (HLA-A2, -A11; -B13, -B46; -Cw1), HB3 (HLA-A2, -A24; -B13, -B60(40); -Cw3), HB4 (HLA-A24, -A33; -B35, -B57(17); -Cw3), and HB5 (HLA-A2; -B38(16), -B61(40); -Cw3), were used as control target cells.

Effector cells. Using HBV-transfected autologous EBV-B cells as stimulators, the intrahepatic $CD8^+$ CTLs specific to HBV were generated by the method described previously (24). Briefly, a piece of fresh nontumor liver tissue was washed extensively with free RPMI (RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone) (GIBCO BRL, Gaithersburg, MD). It was then gently squeezed through a stainless steel mesh to yield a single cell suspension in complete medium (free RPMI supplemented with 5% heat-inactivated human AB serum) (GIBCO BRL). Mononuclear infiltrates were separated from liver cells by Ficoll-Hypaque density-gradient centrifugation. Both liver and mononuclear cells were counted and washed three times with free RPMI. First, 10^3 cells/well of mononuclear infiltrates were cultured in a 96-well flat-bottomed microtiter tray (Costar Corp., Cambridge, MA) in a final volume of 200 μl/well with the following additions: (*a*) 10^3 cells/well of autologous liver cells as transient target or antigen-presenting cells, which may have both HBcAg and HBsAg expression, as revealed by the immunoperoxidase staining (13, 14), and they may also have HLA class I antigen expression on the membrane (16); (*b*) 10^5 cells/well of irradiated (8,000 rad) autologous HBV-transfected EBV-B cells as feeder cells and stimulators; and (*c*) a mixture containing recombinant human IL-2 (20 ng/ml) and IL-4 (1,000 pg/ml) (both from R&D Systems, Minneapolis, MN). After incubation at 37°C in humidified 5% CO_2 atmosphere for 7 d, liver-derived T lymphocyte blasts were harvested, counted, and then cloned by limiting dilutions at 0, 50, 100, 200, 400, 800, and 1,600 cells/well in each plate of 96-well flat-bottomed tray. In each well, we added 1 μg/ml of PHA (Sigma Chemical Co.), 20 ng/ml of IL-2, and 10^5 cells of irradiated autologous HBV-transfected EBV-B cells. Growing cells were expanded in complete medium containing IL-2 and were subjected to HBV-specific cytotoxicity screening assay. Positive clones were further characterized for peptide-specific cytotoxicity activity and phenotype analysis. The responsive clones were expanded with IL-2–containing medium and restimulated with irradiated autologous HBV-transfected EBV-B cells every 2 wk.

Cytotoxicity screening assay. From a large number of T cell lines, CytoTox 96™ nonradioactive cytotoxicity assay system (Promega, Madison, WI) was used for rapid screening of potential CTLs that were responding to HBV-transfected target cells and EBV-B cells pulsed with the naturally occurring peptides. This method was a semiquantitative, calorimetric alternative to the standard ^{51}Cr release cytotoxicity assay. The procedure was performed according to the manufacturer's instructions.

Mass spectrometric analysis

Each active fraction was dissolved in water/methanol (50:50, by vol) containing 1% acetic acid and was checked for the approximate molecular weight and the possibility of the presence of peptide mixtures by a VG platform electrospray ionization mass spectrometry (ESI-

MS) (Fisons VG Biotech; Altrincham, Manchester, UK). The ESI-MS was performed on electrospray-positive mode using the software Mass Lynx 1.6 (Fisons VG Biotech). When the candidate peptide fractions with CTL activity were revealed to be a mixture on the mass spectrum, they were rechromatographied onto the same HPLC column with flatter gradient or isocratic (20% acetonitrile) conditions.

^{51}Cr release assay

After rechromatography, the standard ^{51}Cr release cytotoxicity assay was adopted to identify the active fraction(s), using the same CTL lines selected from the cytotoxicity screening assay described above. Briefly, the peptide fractions were dried and resuspended in 0.5 ml of complete medium. In triplicate, 50 μl of peptide solution was incubated for 90 min with 10^4 cells of ^{51}Cr-labeled autologous or allogeneic EBV-B cells as target cells in a total volume of 150 μl. Target cells were labeled with 0.1 mCi ^{51}Cr (DuPont NEN, Boston, MA) at 37°C for 1 h and were then washed three times with free RPMI before cytotoxicity assay. The appropriate CTLs were added to have an E/T ratio between 10:1 and 40:1 and to have a final volume of 200 μl. After 4 h at 37°C, radioactivity released into the supernatant was determined by a gamma counter. Specific lysis of target cells was calculated using the following formula: (release by CTLs − medium release)/(detergent release − medium release) × 100. Spontaneous release of target cells ranged from 5 to 15%.

Peptide sequencing and synthesis

The peptide fraction(s) with CTL activity were purified and sequenced by Edman degradation on an Applied Biosystems instrument (model 477; Applied Biosystems, Inc., Foster City, CA). Synthetic peptides were prepared by standard Merrifield solid-phase method as previously described (25).

Titration of natural and synthetic peptides

It has been shown the number of empty MHC molecules at the cell surface in overnight culture of mouse tumor cells at 26°C appears to increase (26, 27). The empty class I MHC molecules are easier to bind to antigenic peptide and are stabilized by bound peptide in the presence of exogenous β_2-microglobulin (β_2M) (28–31). We therefore used cool targets to increase empty MHC molecules and human AB serum in the complete medium to supply human β_2M in titration experiments in which target cells were preincubated for 24 h at 26°C before ^{51}Cr labeling.

Database screening

The Geneman protein sequence data bank (DNASTAR, Inc., Madison, WI), which is a combination of SwissProt, PIR/NBRF, and GB-Trans protein databases, was screened for the peptide sequences that were derived from hepatocyte membranes capable of inducing CTL activities.

HBV DNA sequencing

To ensure the identity of the eluted antigenic peptides that may correspond with a sequence of viral origin, HBV DNA fragment was amplified from the patient's liver tissue by PCR and sequenced directly using the dsDNA cycle sequencing system (GIBCO BRL).

Elution of naturally occurring peptides from autologous, HBc-transfected EBV-B cells

Using the same procedures for elution and analysis of liver-derived naturally occurring peptides described above, $\sim 10^{10}$ cells ($\fallingdotseq$ 10 g) of autologous HBc-transfected EBV-B cells were collected for peptide elution and analysis. This was done to confirm that the viral peptides eluted from liver tissue could also be presented and/or bound by the same HLA class I molecules in nonhepatocyte antigen-presenting cells.

HLA-A11 stabilization assay

Various concentrations of synthetic peptides were incubated with the patient's EBV-B cells (10^6 cells), 1 μg human β_2M (Sigma Chemical Co.), and mouse anti–human HLA-A11 mAb (A11.1M; American Type Culture Collection, Rockville, MD). Cells were then washed twice with buffered saline and stained with FITC-conjugated F(ab′)$_2$ fragments of polyclonal goat anti–mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 30 min at 4°C. After two additional washes with buffered saline, cells were fixed with 4% formalin (Sigma Chemical Co.), and flow cytometry was performed on a FACS® (Becton Dickinson & Co., Mountain View, CA). Results were reported as the percentage of increase (over control) of A11.1M mean fluorescence channel number activity. Control represented EBV-B cells cultured with β_2M and A11.1M in the absence of synthetic peptide. The concentration of A11.1M used in the assay was that that had been titrated to be nontoxic to the EBV-B cells. A nonapeptide, amino acid 132–140 of hepatitis C virus core protein restricted by HLA-A2.1 (32), was used as a negative control.

Results

Generation of CTL clones. Using HBV-transfected EBV-B cells as stimulators, seven intrahepatic HBV-specific CD8$^+$ T cell clones (TCCs) were successfully generated by limiting dilution T cell cloning. As shown in Fig. 2, these TCCs had specific CTL activity against autologous HBV-transfected EBV-B cells. These TCCs were used as effectors in the cytotoxicity assay to screen for peptides that could sensitize autologous EBV-B cells to lysis.

Extraction of naturally processed peptides. Acid extracts prepared from liver tissue and HBc-transfected EBV-B cells were separated by HPLC (Fig. 3). There was significant difference on HPLC profiles between each extract. However, a few peaks overlapped on the same chromatographic condition sug-

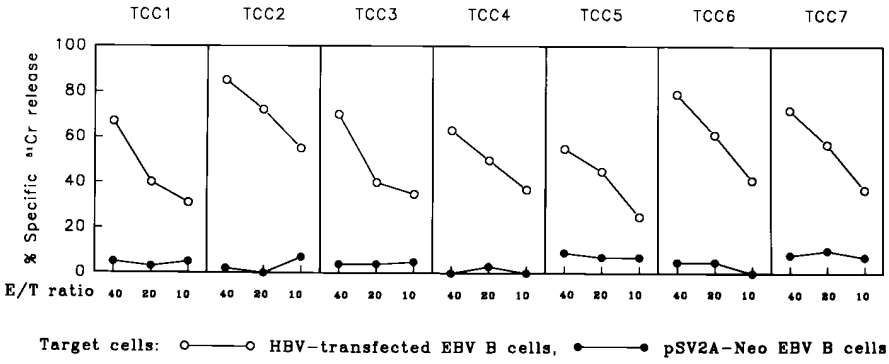

Figure 2. Demonstration of HBV-specific, CD8$^+$ cytotoxic TCCs. TCCs 1–7 generated from intrahepatic cell infiltrates were used as effector cells. Target cells were derived from autologous EBV-transformed B cells transfected by pSV2A-Neo-(HBV)$_2$, which could express HBV proteins including HBsAg, HBeAg, and HBcAg (HBV-transfected EBV-B cells), and transfected by vector only (pSV2A-Neo EBV B cells) as controls.

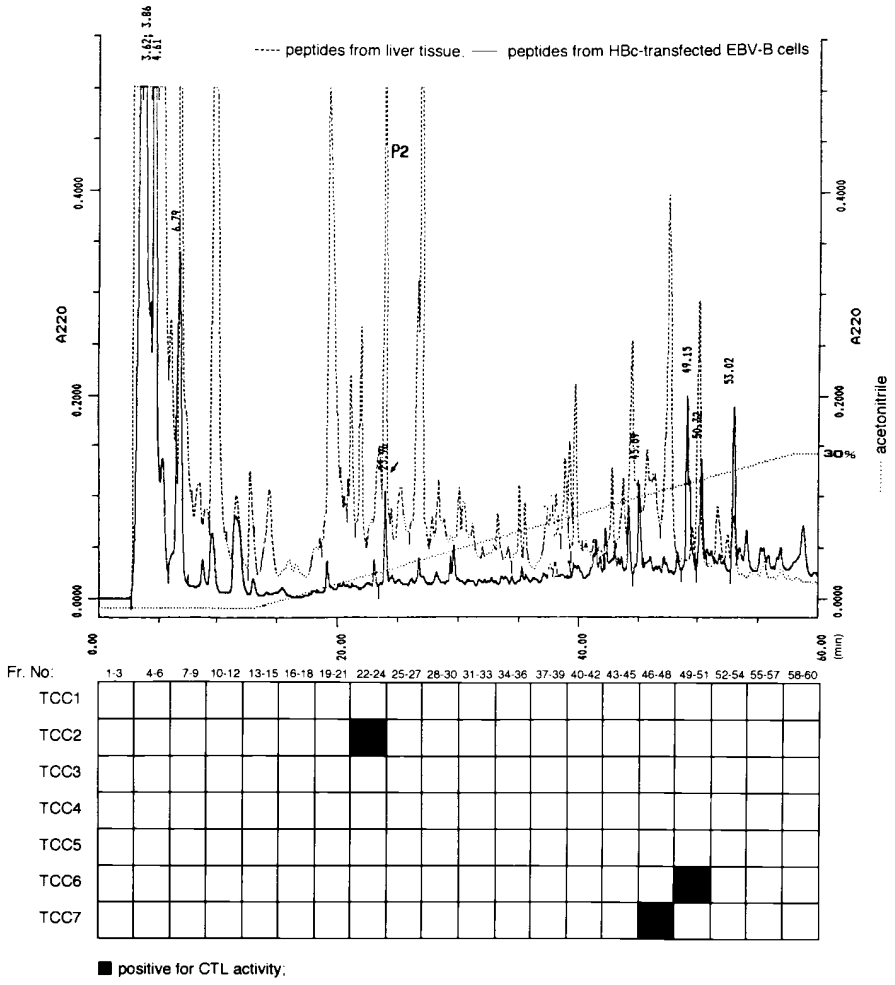

Figure 3. HPLC fractionation of peptides extracted from HBV-infected liver tissue and HBc-transfected EBV-B cells and cytotoxicity screening assay of peptides derived from HBV-infected liver tissue. Low–molecular mass components (< 10 kD) from TFA extraction of adducts of class I MHC molecules were fractionated by reverse-phase chromatography on a C18 column (VYDAC) (*upper panel*). Fractions were collected at 1-min interval (at a flow rate of 1.0 ml/min). For cytotoxicity screening assay, fractions of one run of HPLC were pooled per three fractions; hence, 20 pooled fractions were subjected to the nonisotope cytotoxicity screening assay using autologous EBV-B cells as targets and TCCs 1–7 as effectors (E/T = 3:1) (*lower panel*). Each pooled fraction was lyophilized and reconstituted in 200 μl complete medium. Aliquots of 50 μl of the reconstituted peptide(s) in triplicate were added for sensitizing target cells in each assay. Peptide pools 22–24, 49–51, and 46–48 (*lower panel*) from liver tissue could sensitize target cells to lysis by TCCs 2, 6, and 7, respectively.

gested that similar peptide products might be eluted from both types of tissues.

Cytotoxicity screening assay. In a panel of 20 pooled peptide fractions (one fraction per minute, one pool per three fractions) from liver tissue, the peptide pools 22–24, 49–51, and 46–48 could sensitize target cells to lysis by TCCs 2, 6, and 7, respectively (Fig. 3). Because TCC2 was the best growing one with cycle stimulation by IL-2 and irradiated HBV-transfected autologous EBV-cells, it was selected to screen CTL epitopes. The peptide pool 22–24 in Fig. 3, therefore, was rechromatog-

Table I. Sequencing of One Naturally Occurring Peptide P2 with CTL Activity

	Amino acid residues (in pmol)																		
	D*	N	S	Q	T	G	E	A	H	Y	R	P	M	V	W	F	I	K	L
Cycle	Asp	Asn	Ser	Gln	Thr	Gly	Glu	Ala	His	Tyr	Arg	Pro	Met	Val	Trp	Phe	Ile	Lys	Leu
S‡	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0
1	7.5	10.3	11.8	1.2	9.4	1.7	4.6	6.5	0.0	373.0	0.0	2.2	0.0	2.5	1.9	4.7	3.4	0.0	0.0
2	1.4	8.5	5.6	1.2	3.8	1.6	6.1	2.0	0.0	35.0	0.0	0.0	0.0	104.4	4.1	0.0	0.8	0.0	1.5
3	1.8	142.5	12.6	2.2	8.3	1.3	7.6	2.0	0.0	21.9	0.0	0.6	0.0	14.5	1.4	0.0	1.1	0.2	0.0
4	4.0	50.9	3.8	1.4	1.3	0.7	3.3	1.7	0.3	3.7	0.0	0.6	0.0	110.5	1.7	0.0	0.9	0.0	1.2
5	0.4	117.9	3.6	0.9	2.3	0.7	2.6	1.0	0.0	1.5	0.0	0.0	0.0	8.6	3.4	0.4	0.9	0.4	0.0
6	0.7	11.0	2.4	0.9	1.2	0.4	2.3	2.1	0.5	2.2	6.5	0.2	120.0	6.7	0.8	0.2	1.0	0.0	0.0
7	1.2	0.5	1.6	0.3	1.5	94.5	2.0	1.8	0.0	1.5	0.0	0.0	5.1	3.9	0.2	0.3	1.1	3.5	10.3
8	0.8	0.4	2.0	0.5	0.0	13.3	1.9	1.8	0.4	2.0	0.0	0.3	2.1	1.8	0.5	0.0	0.8	10.0	140.4
9	0.9	0.1	0.1	0.1	2.5	7.2	2.2	1.3	0.1	2.1	2.5	1.0	3.3	1.5	0.5	2.1	7.3	75.1	47.5
10	0.5	0.1	1.1	0.5	0.5	1.0	1.1	0.5	0.4	0.2	0.5	1.2	0.5	0.7	0.3	0.3	1.1	21.0	7.3

Sequence: NH_3-Y-V-N-V-N-M-G-L-K-COOH (HBcAg 88–96), adw or adr subtype of HBV. *The single-letter code for amino acid residues is used, ‡standard.

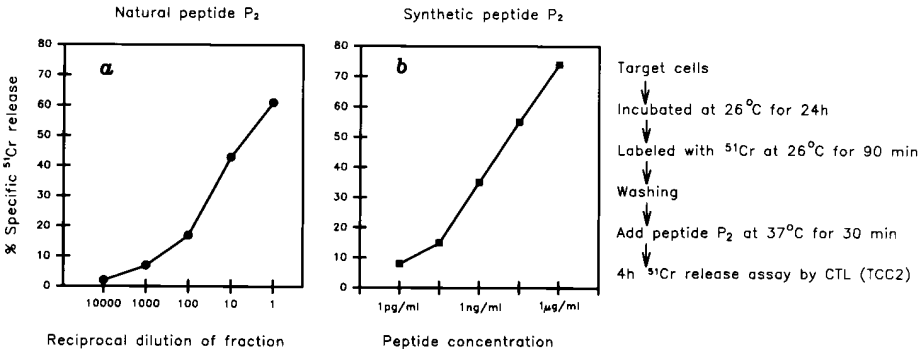

Figure 4. Titration of natural and synthetic peptides. (*a*) Natural peptide P2 purified from one-half of 20 runs of HPLC fractionation was dissolved in 1 ml of complete medium and was tested in titrated concentrations for recognition by TCC2. (*b*) Synthetic peptide P2 was tested in titrated dilution for recognition by TCC2. The experimental procedure using cool target cells is shown at the right side of the figure. Each datum is a mean of triplicate determinations using an E/T ratio of 20.

raphied and collected in 20 fractions (data not shown). Each peptide fraction was further assayed for the CTL activity by TCC2. A single HPLC peak, designated as P2, equivalent to the fraction 24 in Fig. 3 that had the most prominent CTL activity, was subjected to mass spectrometric analysis and peptide sequencing.

Mass spectrometry and microsequencing analysis. The peptide P2 was eluted and analyzed on ESI-MS. It yielded an m/z of 1036.94±0.00 without contamination (data not shown). One-half of the natural peptide P2 collected from 20 runs in total of HPLC fractionation was pooled and lyophilized. It was then subjected to amino acid sequencing. The results shown in Table I revealed that the peptide P2 was a nonapeptide. The overlapped HPLC peak of peptide eluted from HBc-transfected EBV-B cells showed the same molecular weight and amino acid sequence as the peptide extracted from HBV-infected liver tissue. The Geneman protein sequence data bank was screened for this nonamer peptide. It revealed to be derived from amino acid residues 88–96 of HBcAg, which belonged to adw or adr subtype of HBV (Table I).

Titration of natural peptide P2 concentration. As illustrated in Fig. 4, the amount of another half of the natural peptide P2 collected from 20 runs of HPLC that could induce CTL activity in terms of percent specific ^{51}Cr release using cool targets was estimated to be ~ 1 μg. This value approximated reality as calculated in the following.

The current purification protocol yield $\sim$ 0.5–1.0 mg class II MHC molecules from 1 g of EBV-B cells ($\sim$ 1 liter of culture $\approx 10^9$ in cell number). It is estimated that 1 μg of class II MHC molecules contains 16 pmol of extractable peptides, and the efficiency of peptide extraction is in the range of 75–80%. Thus, 1 μg of MHC class II molecules can yield 13 pmol of isolated peptides for the analysis. Because the bound-peptide repertoire is very complex and is estimated to be $>$ 2,000 in number (18), 13 pmol of peptides isolated from 1 μg of class II MHC molecules must be divided by this number to obtain the average molar amount of each individual peptide (18, 19). The amount of extractable peptides from class I MHC molecules was estimated to be comparable to that of class II molecules. In this study, the amount of obtainable peptides from class I MHC molecules of liver tissue could be estimated accordingly: (*a*) amount of liver tissue, 40 g; (*b*) estimated HLA class I expression in HBV DNA–positive liver tissue with chronic hepatitis, 50% in average (16); (*c*) 0.5–1.0 mg HLA class I molecules could be isolated per gram of liver tissue that had full expression of HLA class I antigens; (*d*) 13 pmol bound peptides/μg HLA class I molecules.

The amount of bound peptides extractable from 40 g of liver tissue would be as follows: $a \times b \times c \times d = 40 \times 0.5 \times (0.5–1.0) \times 13 \times 1{,}000 = 1.3 \times 10^5$ to 2.6×10^5 pmol = $(1.3 \sim 2.6) \times 10^{-7}$ mol. This amount divided by 2,000 would still be adequate for functional analysis including CTL assay and peptide sequencing, especially for those predominant peptides that contributed $>$ 20% of total peptides (17). If the peptide P2 contributed 1% (estimated arbitrarily from the HPLC profile in Fig. 3) of total peptides bound to class I MHC molecules on hepatocyte membrane, the amount of peptide P2 purified from 40 g of liver tissue would be as follows: $(1.3 \sim 2.6) \times 10^{-7} \times 1{,}036 \times 10^6$ (mol wt in μg) $\times$ 1% = 1.35 $\sim$ 2.69 μg. One-half of this amount approximated 1 μg.

HBV-DNA sequencing. As the eluted peptide sequence revealed to be derived from the HBcAg 88–96, total DNA was extracted from the patient's liver tissue (nontumor part). PCR amplification of HBV-DNA using specific primers was performed. The PCR product was then directly sequenced and shown in Fig. 5. The amino acid sequences deduced from the HBV-DNA sequence in the liver and the eluted peptide se-

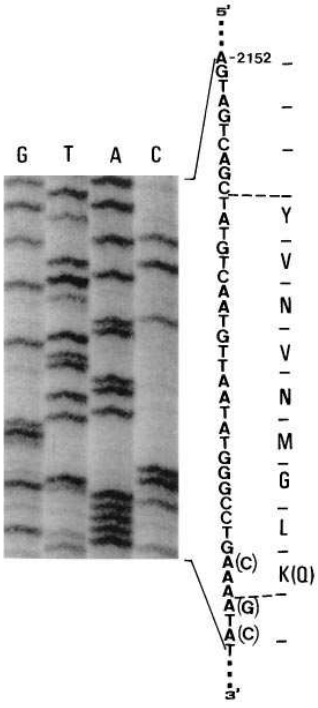

Figure 5. Direct sequencing of HBV-DNA gene segment encoding the P2 peptide from the patient's liver tissue. Total DNA was extracted from the liver tissue by standard phenol-chloroform extraction method. PCR using 5′ primer (5′-CACCACACAGCACTCAGG CAAGCT-3′, nt 2051–2075) and 3′ primer (5′-CGAGGGAGT TCTTCTTCTAGG-3′, nt 2386–2366) proceeded 35 cycles (denaturing at 94°C, 30 s, annealing at 54°C, 60 s, and extension at 72°C, 90 s) in a thermal cycler (Perkin Elmer-Cetus, Norwalk, CT). Subsequently, the PCR product was sequenced directly using ds-DNA cycle sequencing system (GIBCO BRL).

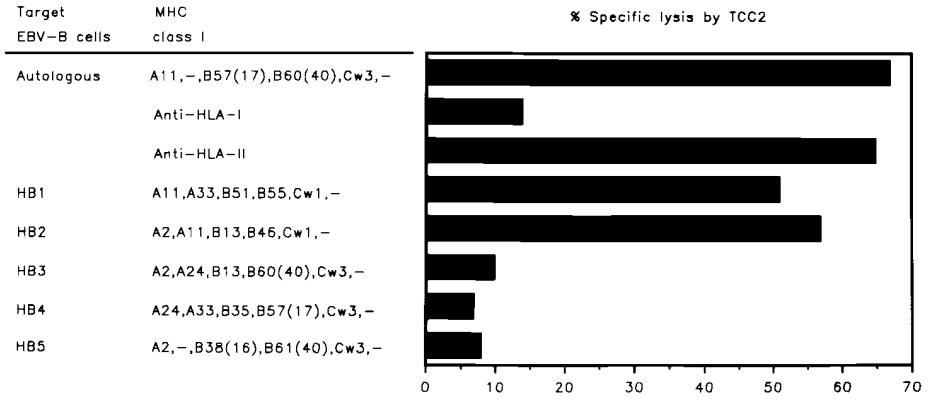

Figure 6. Mapping of the MHC restriction elements for the peptide P2. Autologous and allogeneic EBV-B cells sharing class I MHC alleles were used as the target cells in the cytotoxicity assay. The percentage of specific lysis was assayed at an E/T ratio of 20 and a synthetic peptide P2 concentration of 1 μg/ml. Rat anti–HLA-I (IgG 2a) and anti–HLA-II (IgG 2a) mAbs (both from Serotec, Oxford, UK) were used for the blocking assay at a final concentration of 10 μg/ml in the culture.

quencing were virtually identical, except that a mutation at nucleotide 2186 A to C was found to coexist with wild-type sequence. This base mutation could result in change of amino acid residue from K (Lys) to Q (Gln) (Fig. 5).

MHC restriction of peptide P2. According to the peptide sequence, synthetic nonapeptide was used for a series of cytotoxicity assays using TCC2 as effector cells and EBV-B cells with different HLA class I haplotypes as target cells (Fig. 6). The data revealed that peptide P2 was restricted by HLA-A11 allele.

Peptide P2-specific binding to HLA-A11. Since empty class I MHC molecules could be stabilized by the bound peptide in the presence of exogenous β_2M (28–31), we used this strategy to analyze the ability of peptide P2 to bind to HLA-A11, as it has been used in studies of peptides in the HLAA2–specific stabilization assay (33–35). The results shown in Fig. 7 revealed that the peptide P2 could enhance significantly the reactivity of HLA-A11–positive cells with the A11.1M (anti–HLA-A11 mAb) compared with the irrelevant peptide hepatitis C virus core 132–140. This evidence strongly supported the theory that the nonapeptide P2 purified from the extract of HBV-infected liver cell membrane was actually HLA associated and had not just been released by chance from the HBV particles in liver cells through the homogenization of liver tissue.

Discussion

We have identified a naturally processed peptide P2 derived from HBV nucleocapsid antigen or HBcAg. The P2 peptide was isolated from MHC class I complexes of HBV-infected liver cell membranes, and it could also be eluted from HBc-transfected EBV-B cells. The amino acid sequence of P2 (HBcAg 88–96; YVNVNMGLK) obtained from direct sequencing of HPLC elute was concordant with that deduced from the viral genome isolated from the liver. The P2 epitope could be recognized by HLA-A11–restricted, $CD8^+$ CTL isolated from the patient's liver infiltrates. The P2 epitope shared the HLA-A11 binding motifs with amino acid residues Val (V) at position 2 and Lys (K) at position 9 of the nonapeptide. The peptide P2 exhibited the ability to bind to HLA-A11 as revealed by the HLA-A11–specific stabilization assay. These data provided direct evidence that, in hepatitis B patients, antigenic peptides of HBV were processed and could bind to MHC class I molecules, which were in turn presented on hepatocyte membranes. The MHC class I–peptide complexes were capable of being recognized by $CD8^+$ CTLs that may trigger hepatocytolysis and may lead to elimination of infected cells. This may be one of the prime mechanisms of host immune surveillance against HBV infection (4).

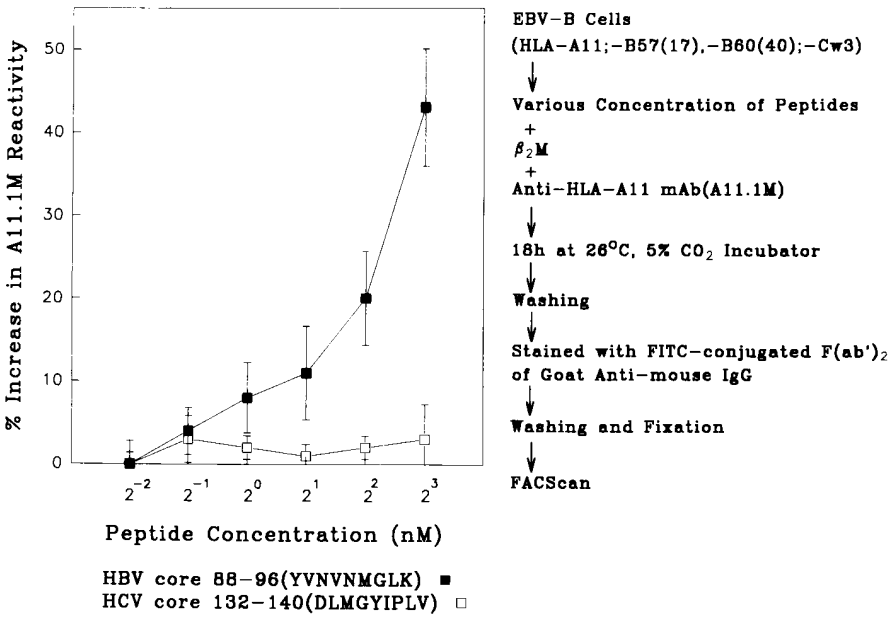

Figure 7. HLA-A11 stabilization assay. The assay procedure is shown at right side of the figure. HLA-A11 expression was detected using the A11.1M in the indirect immunofluorescence assay monitored by flow cytometry. Data are reported as percent increase in A11.1 M (anti–HLA-A11 mAb) reactivity relative to autologous EBV-B cells incubated in the absence of peptide. Each datum is a mean of triplicate determinations.

In terms of MHC class I–restricted recognition, in vitro studies have shown that HBcAg- and HBsAg-specific $CD8^+$ CTL lines or clones can be generated from acute and chronic hepatitis B patients (5–12, 24, 36, 37). The recognition was based on the demonstration of lysis of HBcAg-transfected or HBsAg-transfected EBV-B cells by CTLs. The virus-specific CTL activities can also be detected by addition of synthetic peptides to MHC class I–compatible target cells (6, 9, 11, 24, 37), as it has been well characterized in defining the epitopes of influenza nucleoprotein recognized by CTLs (38). Similarly, the blocking of virus-specific CTL responses can be demonstrated by addition of mutant peptides derived from naturally occurring variant viruses (39). In this study, we have shown that there is a naturally occurring HBV peptide capable of sensitizing target cells to lysis by $CD8^+$ CTLs. This confirmed the validity of those experiments using EBV-B cells as target cells in the exploration of CTL mechanisms of HBV immunopathogenesis (for review see reference 4).

In terms of MHC class II–restricted recognition by $CD4^+$ T cells, Chisari et al. (4) and Milich et al. (40) have conducted extensive studies in the human system and in the murine model, respectively, and have analyzed the immune response to HBV proteins thoroughly. Although it is not addressed in this report, we have begun a similar study on the class II–restricted natural peptides eluted from HBV-transfected EBV-B cells and on the generation of relevant $CD4^+$ T cell lines/clones.

Since MHC class I–restricted CTLs have potent antiviral activity, persistence of virus could almost be defined as the virus that has evolved some kind of mechanism for evading the CTL response of the host (for reviews see references 41, 42). One such mechanism for escape from CTL recognition may involve genetic variation, as demonstrated in HIV, influenza virus, lymphocytic choriomeningitis virus, EBV, and HBV (for review see reference 42). In chronic HBV infection, Chuang et al. (43) have reported that there are three mutation clustering regions (MCR) in core gene, codons 48–60 (MCR1), 84–101 (MCR2), and 147–155 (MCR3). MCR2 is the most remarkable, having the highest mutation rates studied in 20 chronic active hepatitis B patients irrespective of their HLA haplotypes (44). This suggests the possibility that MCR2 carries important T cell recognition epitope(s). In this study, the isolation of naturally occurring HLA-A11–restricted CTL epitope P2 within the MCR2 strongly supports this possibility and may be clinically relevant to patients in Southeast Asia, including Taiwan, where the A11 allele is present in $> 50\%$ of individuals (45). Furthermore, it is noteworthy that a mutation located at HLA-A11 anchor residue in position 9 (codon 96, K→Q) of the nonapeptide P2 was found to coexist with wild-type virus in the liver tissue. This may cause A11 epitope loss as demonstrated in the T cell epitope of EBV-encoded nuclear antigen 4 (amino acid residues 416–424) (45). Investigation of the possibility that the naturally occurring HBV variant may be a T cell receptor antagonist for antiviral CTLs is ongoing in our laboratories.

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