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Identification and functional studies of HLA-A0201 restricted CTL epitopes in the X protein of hepatitis B virus

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Summary. – Cytotoxic T lymphocyte (CTL) epitopes in the X protein (HBx) of hepatitis B virus (HBV) may play a key role in the viral control and liver damage. The aim of this study was to identify and study the function of HLA-A0201 restricted CTL epitopes in HBx of HBV genotypes B and C that are epidemic in China. Four nonapeptides signed HBx1: VLCLRPVGA, HBx2: CLFKDWEEL, HBx3: VLHKRTLGL, and HBx4: HLSLRGLPV were predicated by computational analysis and manually confirmed by defining the peptide supermotif, extended motif, and quantitative motif. Synthesized peptides were examined for their affinity and binding stability with HLA-A0201. After being analyzed by enzyme-linked immunospot (ELISPOT) and cytolytic activity assays, the HBx2 epitope was selected for a construction of HLA-A0201-peptide tetramers. The tetramer staining method was used to analyze peripheral blood mononuclear cells (PBMCs) isolated from HBV-infected patients at different disease stages (chronic hepatitis, liver cirrhosis, and hepatoma). Compared with CTL epitopes in the HBV envelope or polymerase, HBx2 is also a potential HLA-A0201 restricted CTL epitope, what may have a clinical implication.

Keywords: hepatitis B virus; HLA-A0201 antigen; T-cell epitope; cytotoxic T lymphocyte

Introduction

HBV infection is a serious disease with a worldwide distribution (Kremsdorf *et al*., 2006; Bruss, 2004). According to the Ministry of Health of the People′s Republic of China, hepatitis B infection is ranking second among the common infections in China. Sequence variation of the HBV genome is associated with the virus infection and immune evasion. HBV sequence data are used to trace down routes of infection, reconstruct the phylogenetic history of viruses, and develop anti-virus strategies (Magnius and Norder, 1995). Hepatitis B surface antigen (HBsAg) has five major subtype determinants termed a, d, y, w, and r, which have primarily epidemiologic significance. All HBsAg-positive sera contain the determinant a. The determinants d and y are mutually exclusive as are w and r. Hence, four subtype patterns are possible, e.g. adw, ayw, adr, and ayr. HBV can also be classified into eight genotypes signed A, B, C, D, E, F, G, and H (Okamoto *et al*., 1988). The different genotypes may have a different pathogenicity. Genotypes B (adw) and C (adr) are the most prevalent in China (Usuda *et al*., 1999; Kao *et al*., 2000; Liu *et al*., 2002; Xu *et al*., 2003).

The HBV contains partial double-stranded circular DNA of 3.2 kb containing four ORFs. HBx encoding sequence is the smallest ORF (Bouchard and Schneider, 2004). HBV infection can result in the hepatocellular carcinoma (hepatoma) and many studies suggest that HBx gene plays an important role in this disease (Murakami, 2001; Bouchard and Schneider, 2004; Tang *et al*., 2006). The multifunctional HBx is necessary for transcription of virus genome and it could affect a variety of physiological events potentially involved in

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Abbreviations: β2m = β2 microglobulin; CTL = cytotoxic T lymphocyte; ELISPOT = enzyme-linked immunospot; FACS = fluorescence activated cell sorter; FI = fluorescence index; HBc = hepatitis B core; HBsAg = hepatitis B surface antigen; HBV = hepatitis B virus; HBx = HBV X protein; HCC = hepatocellular carcinoma; IFN = interferon; LDH = lactate dehydrogenase; MAb = monoclonal antibody; PBMCs = peripheral blood mononuclear cells; TAP = transporter associated with antigen processing

the development of neoplasia such as the virus replication, mismatch repair, cell kinetics, and apoptosis (Murakami, 2001; Kremsdorf *et al.*, 2006). In hepatoma patients infected with HBV the expression of HBx is higher than the expression of other viral proteins (Tang *et al.*, 2006). Thus, research of this protein could have a clinical significance.

A precise epitope identification especially of the CTL epitopes that can effectively combine with MHC class I could be critical for the molecular diagnosis and biotechnology applications (Hagmann, 2000). Although several reports have described the epitopes of HBx (Chung *et al.*, 1999; Hwang *et al.*, 2002; Malmassari *et al.*, 2005), the analysis of peptide-specific CTL responses in large cohorts of HBV-infected patients have rarely been reported.

In the present study, we identified and characterized distinct HLA-A0201-restricted CTL epitopes of HBx using the most prevalent sequences of HBV in China (genotypes B and C). HBx peptide-MHC-tetramer was constructed to analyze the epitope peptide-specific $CD8^+$ T-cells in HBV-infected patients, who were grouped according to the disease stage, e.g. chronic hepatitis, liver cirrhosis, or HCC. Thus, peptide-MHC-tetramer based assay was developed for the potential accurate clinical diagnosis for HBV-related liver disease.

Materials and Methods

Cell line and patients. Human T2 cells deficient in transporter associated with antigen processing (TAP) were obtained from the American Type Culture Collection. HBV-infected $HLA\text{-}A0201^+$ patients (n = 73) were allocated into three groups according to the disease stage: chronic hepatitis (n = 27), liver cirrhosis (n = 21), and HCC (n = 25). The other group of 22 $HLA0201^-$ patients served as control. Patients with HBV infection were recruited from the Hepatitis clinic at the Changhai Hospital and the Eastern Institute of Hepatobiliary Surgery (Shanghai, China) between January 2008 and March 2009. Patients showing co-infection with hepatitis C, hepatitis D, pregnant women, or asymptomatic carriers were excluded. Chronic hepatitis B was defined by detectable levels of serum HBsAg and alanine aminotransferase that were 2 times higher than the upper limit found in normal serum. This requirement should be met at least in two assays performed 6 months apart. Diagnosis of liver cirrhosis was based on the morphological and clinical criteria, laparoscopy, ultrasound, computed tomography, or angiography according to the standard definitions (Leevy *et al.*, 1994). HCCs (incident cases) were proved by histology. As a control group, 19 $HLA\text{-}0201^+$ healthy subjects (without evidence of infection of HBV) chosen from a large random panel of more than 300 HLA-typed healthy individuals were recruited from the Shanghai Blood Center. The study was approved by the local government ethical committee and the consent was obtained from all patients.

HBV sequence. We selected serotype adw of HBV/B (NCBI Protein Acc. Nos. AAL49992, ABK13705, ABK27214, and ABK27207) and serotype adr of HBV/C (Acc. Nos. AAL07390, AAW03312, BAC65198, and BAC65197) as candidate sequences.

Prediction and synthesis of HBx peptides. A variety of computational tools are now available for predicting T cell epitopes (Leevy *et al.*, 1994). We analyzed the potential peptides of HBx for binding to the human HLA-A0201 molecules by BIMAS (Parker *et al.*, 1994), SYFPEITHI (Rammensee *et al.*, 1999), and ProPred-1[22] algorithms. Four peptides generated by these methods were also manually checked by defining the supermotif (Doytchinova *et al.*, 2004), extended motif (Ruppert *et al.*, 1993), and quantitative motif (Mallios, 1999). Additionally, an HBV-restricted CTL epitope ($HLA\text{-}0201^+$: FLPSDFFPSI) from human hepatitis B core (HBc) was used as a positive control and an irrelevant peptide RLRDLLLIVTR derived from human immunodeficiency virus (HIV) as a negative control. These six peptides were custom-synthesized (Biotech Bioscience & Technology, Shanghai, China) and purified by reverse-phase HPLC chromatography with a purity of more than 95%.

T2 cell binding assay. T2 cells were incubated in DMEM and supplemented with 10% fetal bovine serum (FBS) for 18 hrs at 37°C in 5% CO_2. Cells were collected and washed three times in PBS and incubated in a 24-well plate. Cells used for positive and background (no peptide) controls were grown separately. 2×10^5 cells were incubated with 50 μmol/l candidate peptides and 2.5 μg/ml human β2-microglobulin in serum-free DMEM for 18 hrs at 37°C in 5% CO_2. Then, the cells were collected and again washed three times before being incubated with 2 μl/well of FITC-conjugated anti-HLA-A0201monoclonal antibody (MAb) BB7.2 for 10 mins at room temperature. The cells were washed with PBS and fluorescence data were collected and analyzed by FACScalibur and CellQuest Pro. The voltage of flow cytometry FACSCalibur was adjusted to revise the peak of fluorescence values of positive control group and background control group, e.g. positive $>10^1$ and background $<10^1$. Next, the fluorescence data of different groups were acquired by collecting of 5,000 T2 cells. All experiments were performed in triplicate and the mean determined. Using a special analytical program (CellQuest Pro) we analyzed the fluorescence values of the different groups and computed the fluorescence indexes to weigh the binding affinity of each of the four candidate peptides. The affinity between peptides and HLA0201 was expressed as a fluorescence index (FI) and calculated as FI = (mean fluorescence with the given peptide – mean fluorescence without peptide) / (mean fluorescence without peptide). The value FI >1 indicated a high affinity.

T2 cell binding stability assays. T2 cells were collected and spread in a 96-well plate and incubated with the candidate peptide (50 μmo/l) and human β2-microglobulin (β2m, 2.5 μg/ml) in serum-free DMEM for 18 hrs at 37°C in 5% CO_2. The excess of peptides was washed off and serum-free DMEM with Brefeldin-A (10 μg/ml) for the inhibition of expression of HLA-A0201 on the surface of T2 cell membranes was added into each well. The cells were incubated for 1 hr at 37°C in 5% CO_2, collected after 0, 2, 4, 6, or 8 hrs and incubated with FITC-conjugated anti-HLA-A0201 MAb BB7.2 (2 μl/well) for 10 mins at room temperature. For samples collected at the

different time of incubation, T2 cell expression of HLA-A0201 was calculated using the formula: peptide-induced HLA-A0201 expression = mean fluorescence of peptide-preincubated T2 cells – mean fluorescence of T2 cells treated in similar conditions in the absence of peptide. All experiments were performed in triplicates.

In vitro induction of CTLs in PBMCs from HBV-infected patients with HBx-derived peptides. To generate peptide-specific CTLs, PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation from HLA-A0201$^+$ patients with HBV infection. After lysis of the erythrocytes, the cells were incubated with 10 μg/ml of each peptide in a 24-well plate. The culture medium consisted of 45% RPMI-1640, 45% AIM-V medium, 10% FBS, and 100 U/ml penicillin. On days 3 and 10, half volume of the culture medium was removed and replaced with a fresh medium containing 40 U/ml of human interleukin-2 (IL-2). On day 7, the cultures were re-stimulated with the peptide (10 μg/ml) and IL-2 (20 U/ml). The HBx peptide-stimulated PBMCs were further cultured with IL-2 (20 U/ml) and irradiated allogenic HLA-A0201$^+$ positive feeder cells for 21 days. Then, the cytoxicity assay was performed.

Interferon (IFN)-γ ELISPOT assay. A 96-well plate was coated with 5 μg/ml anti-IFN-γ MAb (BD PharMingen) in 50 μl of 50 mmol/l bicarbonate buffer (pH 9.6) overnight at 4°C. The wells were blocked with 200 μl of 1% BSA made in PBST (PBS with 0.05 % Tween 20) at room temperature for 2 hrs and washed three times with serum-free medium. The coated wells were filled with PBMCs isolated from HLA-A0201$^+$ patients with HBV-infection in amount 1×10^5 cells/well in complete DMEM and incubated for 40 hrs at 37°C in 5% CO_2 with homologous peptides or a negative peptide (2 μg/ml). After incubation, the wells were washed three times with PBST before 50 μl of 0.5 μg/ml biotin-conjugated anti-

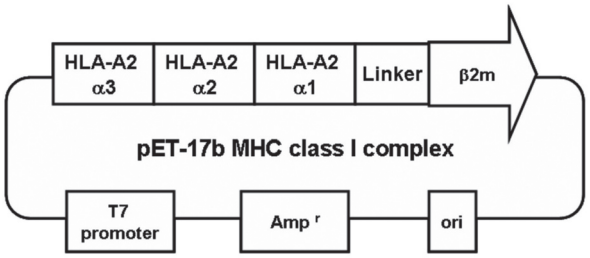

Fig. 1

Schematic representation of pET17b MHC class I complex expression plasmid

The arrow indicates the sequence encoding chimeric MHC class I complex.

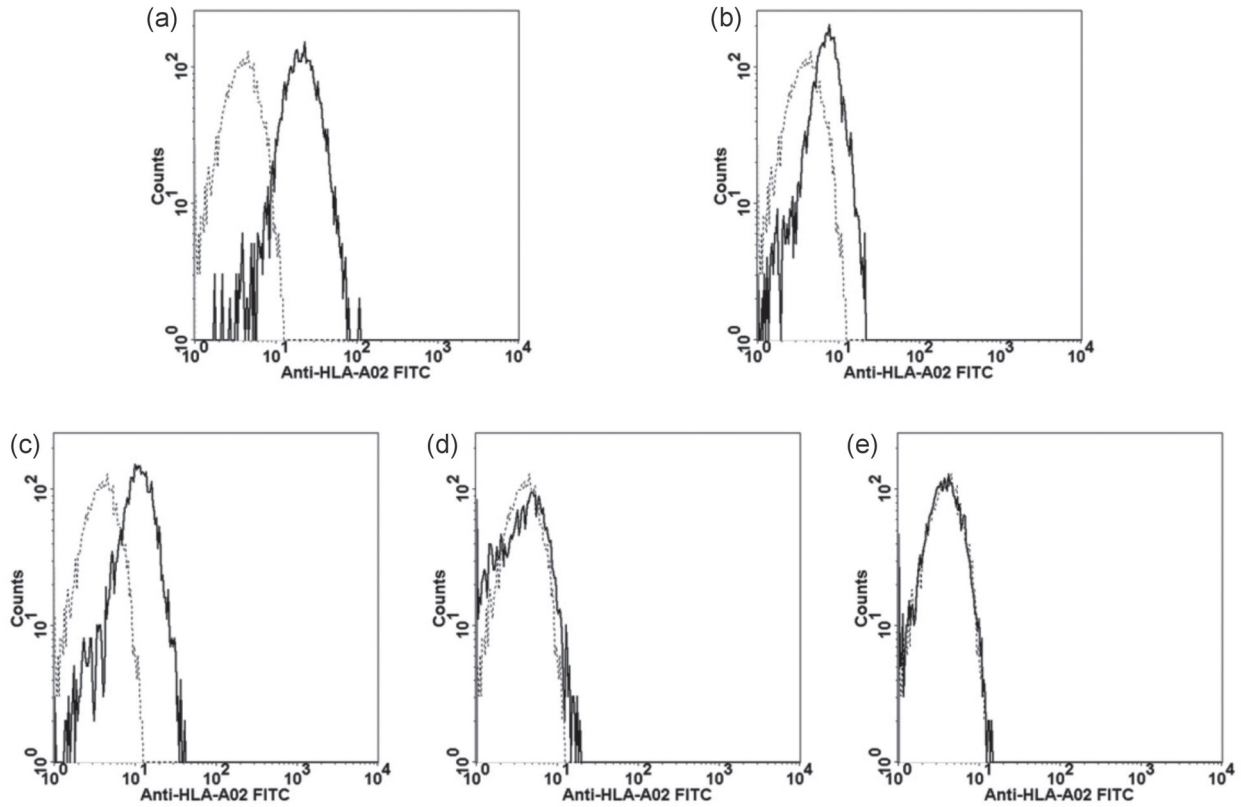

Fig. 2

Flow cytometry analysis of experimental and control groups in a T2-binding assay

Solid curve represents the positive control (a), HBx1 (b), HBx2 (c), HBx3 (d), and HBx4 (f). Dotted curve represents the negative control.

Table 1. List of candidate peptides

Designation	Subsequence	Position	Ranking*			Genotype	Serotype
			a	b	c		
HBx1	VLCLRPVGA	15–23	6	6	11	C/B	adr/dw
HBx2	CLFKDWEEL	115–123	1	1	3	C/B	adr/adw
HBx3	VLHKRTLGL	92–100	3	2	2	C	adr
HBx4	HLSLRGLPV	52–60	10	10	5	C/B	adr/adw

*Ranking of peptide was separately appraised by computational analysis. a: http://bimas.dcrt.nih.gov/molbio/hla_bind/; b http://www.imtech.res.in/raghava/propred1/index.html; c: http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm.

IFN-γ MAb (BD PharMingen) was added and left for 90 mins at room temperature. The plates were washed again and alkaline phosphatase-conjugated streptavidin was added for 1.5 hr. After washing three times with PBST and two times with PBS, the spots were developed by adding substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. After the appearance of spots (up to 1 hr), the reaction was stopped with tap water and the samples were air-dried. An automated ELISPOT counter was used to count spots. The median number of spot-forming cells in three wells was determined. Responses greater than five spots/well and two times the maximum background were considered positive. The final results were expressed as a specific median number per 1×10^5 CTLs after background subtraction.

Peptide-specific CTLs activity assay. The cytotoxicity of peptide-specific CTLs was measured by lactate dehydrogenase (LDH) release assay as described previously (He *et al*., 2005). Target cells were prepared by incubating T2 cells with candidate peptides or a negative peptide (10 μg/ml) (RLRDLLLIVTR) for 4 hrs. T2 cells were also used as background controls. Peptide-specific CTLs as effectors were mixed with peptide-labeled or empty T2 cells in different effector/target ratios (100:1, 50:1, or 20:1). After the DMEM medium was replenished to 200 μl, the cell mixtures were co-incubated at 37°C in 5% CO_2 for 4 hr. The supernatant in volume of 50 μl of was taken out, added to the LDH substrate, and the values A_{490} were taken. All experiments were performed in triplicates and the results were expressed using the formula: % specific lysis = [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100.

Expression of fusion MHC-β2m peptide tetramer and staining of PBMCs. The genes of human MHC heavy chain and β2m were tandem-cloned into plasmid pET17b and expressed as a fusion protein (Fig. 1). The human β2m-HLA-A0201-BSP fusion gene was constructed by RT-PCR and PCR. DNA encoding 14 aa peptide recognized by the biotinylation enzyme BirA was linked to the C-terminus of the extracellular region of HLA-A0201 heavy chain. HLA-0201 α chain β2m was expressed in the form of the inclusion bodies in bacteria. The inclusion bodies were washed and solubilized in 8 M urea. Then, the denatured fusion protein was refolded with peptide in a refolding buffer consisting of 100 mmol/l Tris-Cl (pH 8.0), 400 mmol/l L-arginine, 2 mmol/l EDTA, 5 mmol/l reduced glutathione, 0.5 mmol/l oxidized glutathione, and 0.5 mmol/l phenylmethylsulfonyl fluoride. The re-folding mixture was stirred and incubated at 4°C for 24 hrs. Next, the mixture was desalted and the buffer was changed to the 20 mmol/l Tris-Cl buffer by ultrafiltration with a 30,000 MWCO membrane (Amicon). The folded product was biotinylated using BirA enzyme at 25°C for 18 hrs and then purified by gel filtration. HLA-peptide tetramers were created by mixing biotinylated MHC with phycoerythrin-labeled streptavidin at a molar ratio of 4:1.The tetramers were purified by gel filtration, concentrated to 3 mg/ml of total protein, and stored at 4°C.

PBMCs were obtained from the blood samples of patients and controls. The cells (2×10^5) were stained at a concentration of 0.1 mg/ml of tetramers and FITC-conjugated anti-CD8 MAb at 37°C for 15 mins. After being washed twice with PBS, the stained cells were fixed with 0.5% paraformaldehyde before analysis with FACScan (Wang *et al*., 2004).

Statistical analysis. The biological parameters of HLA-A2 positive or negative patients were analyzed with the χ^2-test. Statistical comparisons between experimental and control groups were performed using one-way ANOVA and Dunnett's method with $P < 0.05$ regarded as a statistically significant difference. Data were presented as means ± SD.

Results

Prediction of HBx peptides

The concordant epitopes identified by all the algorithms were selected as candidate peptides. Four nonapeptides were selected from HBV protein sequence data by three computational analyses based on network services and subsequently confirmed by supermotif, extended motif, and quantitative motif definition (Table 1).

Analysis of T2-HLA-A0201 binding affinity

Expression of HLA-A0201 on the surface of T2 cell membranes was examined by staining with MAb FITC-BB7.2 and FACS analysis. Fluorescence data were acquired by collecting of 5,000 cells from each of the different experimental groups and the FI was analyzed by CellQuest Pro (Fig. 2). After performing this experiment for three times, the affinity between peptides and HLA-A0201 was measured according

Table 2. Expression levels of different HLA-A0201/peptide complexes

Designation	HLA/peptide complexes detected in affinity assay (hrs)					DC50 (hrs)
	0	2	4	6	8	
HBx1	1.88	0.51	0.24	0.18	0.15	1.53
HBx2	2.26	1.28	0.81	0.79	0.77	2.29
HBx3	0.93	0.35	0.22	0.15	0.13	1.80
HBx4	0.35	0.20	0.11	0.07	0.04	2.51
Positive peptide	5.28	3.87	3.17	2.71	2.44	6.49

to their FI. The values for the peptides HBx1-HBx4 were 0.5495, 1.1164, 0.252, and 0.2135, respectively. The positive peptide showed a value 2.21336 and the control background value was 0.2102. The peptide HBx2 that had FI greater than 1 was regarded as a high-affinity epitope. Other peptides HBx1, HBx3, and HBx4 showed FI value less than 1 and were regarded as low-affinity epitopes.

Analysis of peptides binding stability

To analyze the peptide binding stability, median fluorescence intensity was calculated separately for each experimental group (Table 2). Peptide-induced HLA-A0201 expression was determined using the formula described above. The instant dissociation of different MHC-peptide complexes was exhibited by a continuous curve and the dissociation time of 50% MHC-peptide complexes (DC50) was obtained (Table 2). DC50 was defined as the time required for the loss of 50% of the maximal fluorescence value stabilized at time 0 hr. The value DC50 > 2 hrs was used to define a high stability of peptide complex. Peptides HBx2 and HBx4 showed a high stability and peptides HBx1 and HBx3 a low one. Taking into account the results of the T2-binding assay and DC50 assay, the peptide HBx2 was considered as a suitable candidate for HLA-A0201-restricted CTL epitope.

Detection of IFN-γ responses of peptide-specific CTLs

IFN-γ secretion from the peptide-specific CTLs of patients is indicated in Fig. 3. The number of cells secreting IFN-γ in the peptide-specific CTLs (re-stimulated with the homologous peptide) was significantly (P <0.05) higher than the corresponding negative control (re-stimulated with the irrelevant peptide). This outcome suggested that the selected HBx peptides could act as functional epitopes and activate T cells.

Detection of cytotoxicity of peptide-specific CTLs

Peptide-specific CTL responses induced by candidate peptides were determined by LDH release assay. HBx2 peptide-induced CTLs from HLA-A0201$^+$ patients with HBV infection specifically lysed the HBx2 peptide-loaded T2 cells in an effector cells/ target cells (E/T) ratio-dependent manner (Fig. 4). However, the CTL responses induced by the other peptides were non-significant (P >0.05). The negative control did not induce a CTL response and spontaneous release was below 10% of the maximum release. These results indicated that HBx2 is the epitope with a high immunogenicity.

Detection of HBx-specific HLA-A0201-peptide tetramer$^+$/ CD8$^+$ CTLs in PBMCs

The peptide HBx2 was selected to construct a HLA-A0201-peptide tetramer. At least 5×10^4 PBMCs were collected and analyzed for CD8$^+$ CTL expression and tetramer staining. HBV-infected HLA-A0201$^+$ patients (n = 73) were grouped according to the different disease stages (chronic hepatitis 27, liver cirrhosis 21, and hepatoma 25). Furthermore, HLA-A0201$^-$ patients with HBV infection (n = 22) and HLA-A0201$^+$ healthy subjects (n = 19) were also tested

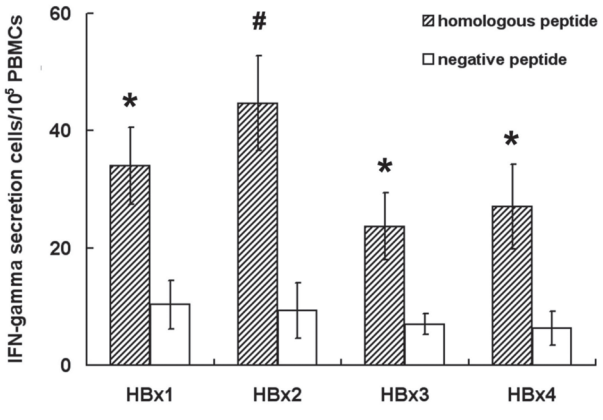

Fig. 3

IFN-γ secreting PBMCs stimulated by different peptides

Peptide-specific CTLs were re-stimulated with homologous and negative peptide. Each bar represents the mean ± SD of IFN-γ secreting cells from three independent experiments. $^{\#}P$ <0.01 vs the corresponding negative control. $^{*}P$ <0.05 vs the corresponding negative control.

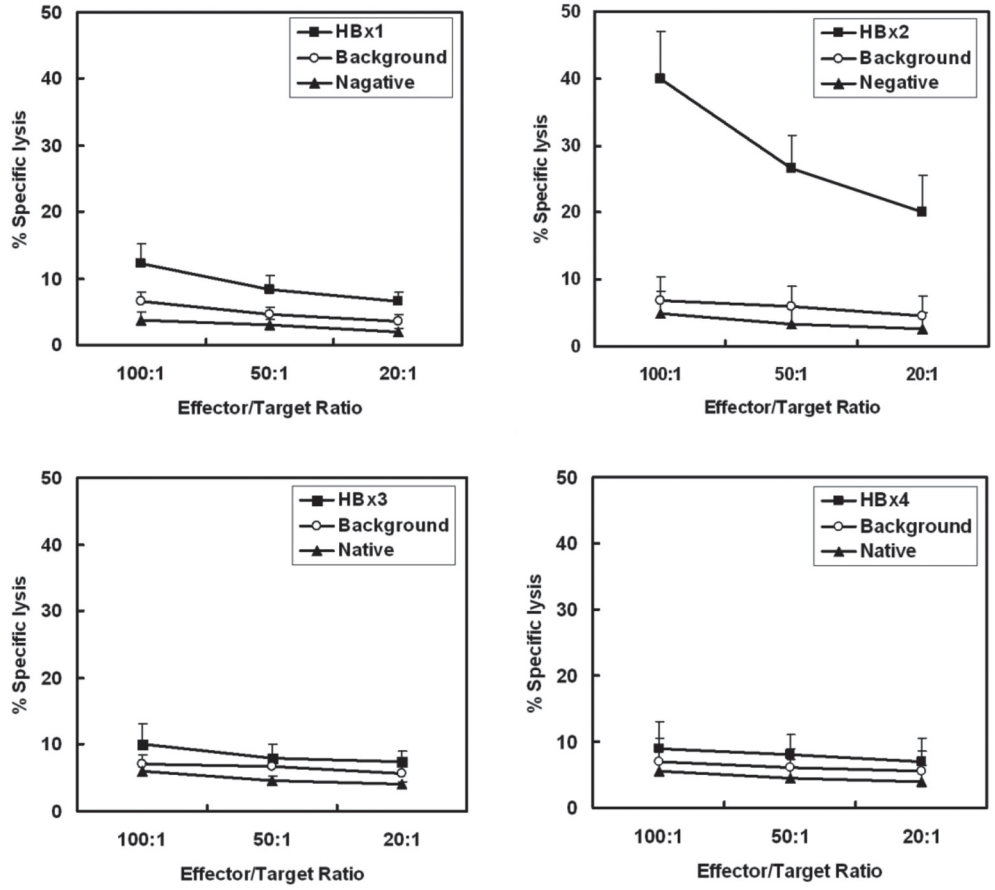

Fig. 4

Cytotoxicity of peptide-specific CTLs stimulated by peptides HBx1 – HBx4

Cytotoxic activity of CTLs was assessed by LDH-release assay at the Effector/Target ratio = 100:1, 50:1, and 20:1.

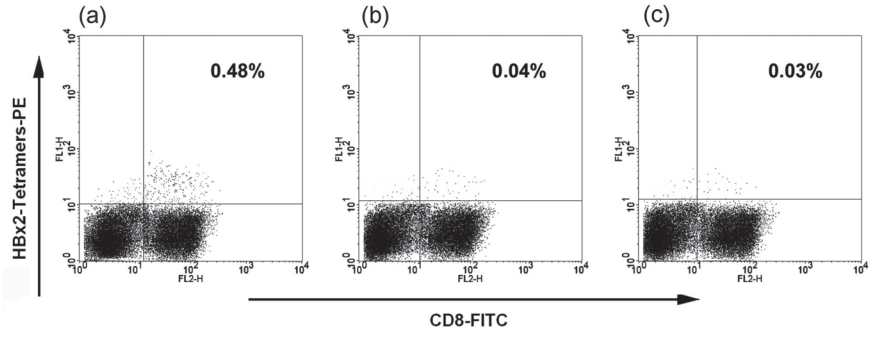

Fig. 5

Flow cytometry analysis of HBx2-specific tetramer$^+$/CD8$^+$ CTLs in peripheral blood

Dot plots of PBMCs stained with the HBx2-tetramer and anti-CD8 MAb. The numbers indicated in the upper-right corner represent the percentage of tetramer$^+$ cells in the CD8$^+$ CTLs subset calculated with CELLQuest™. The representative results are shown for HBV-patient with HLA-A0201$^+$ (a), HBV-patient with HLA-A0201$^-$ (b), and a healthy donor with HLA-A0201$^+$ (c).

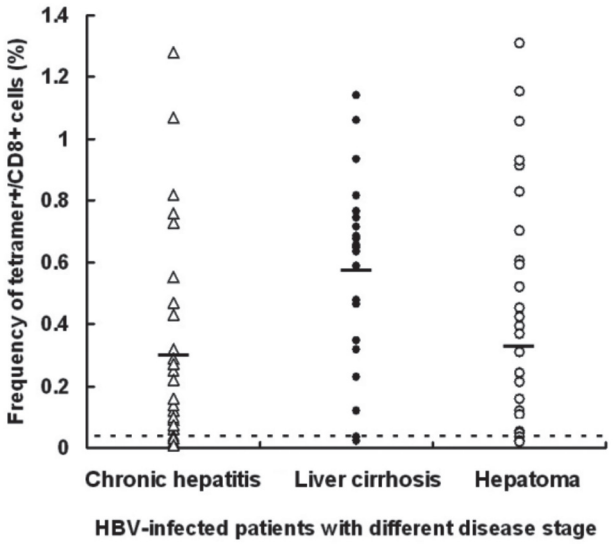

Fig. 6

Quantification of HBx2-specific tetramer+/CD8+ cells in the blood of HBV-patients

PBMCs were stained with HBx2-specific tetramer and anti-CD8 MAb. The frequency of tetramer$^+$ CD8$^+$ cells was calculated from 10,000 CD8$^+$ cells analyzed. The dash line is the cutoff value obtained from control patients and HLA-A2$^+$ healthy donors. The short solid line indicates the mean for each group.

as negative controls. The frequency of tetramer-positive cells was less than 0.04% of circulating CD8$^+$ cells that was the maximal value observed in the controls (Fig. 5). The frequency of tetramer-binding cells was different in the patient groups (Fig. 6). The HBx epitope specific for CD8$^+$ cells were higher than the background in 21 of 27 patients with chronic hepatitis, in 19 of 21 patients with liver cirrhosis, and in 20 of 25 patients with hepatoma. The frequency of tetramer$^+$/CD8$^+$ cells in patients with liver cirrhosis was significantly higher (P <0.05) than that in the patients with chronic hepatitis or hepatoma. There was no obvious difference between the latter two groups (P >0.05). This demonstrated that the HBx-specific CD8$^+$ T cell responses was present in most HBV-infected patients and that the HBx epitope HBx2 was an ideal epitope that could be used in the clinical diagnosis.

Discussion

We analyzed CTL epitopes in HBx in China (genotypes B and C). Potential HLA-A0201$^+$-specific binding peptides within the target sequences were initially predicted using the three computer programs. This approach resulted in four candidate peptides HBx1, HBx2, HBx3, and HBx4 that were confirmed manually by supermotif, extended motif, and quantitative motif definition. Many researchers suggested that not all the peptides predicted by computer algorithms would be valuable for the immunological identification (Andersen *et al.*, 2000; Nussbaum *et al.*, 2003). Thus, measuring specific CTL responses is necessary to select a functional CTL epitope.

We examined the affinity of predicted peptides for the binding stability with HLA molecules using the T2 binding assay and DC50 assay. In the T2 binding assay, the highest FI value of the four candidate peptides was obtained for HBx2 (>1), what indicated that the HBx2 epitope had the strongest affinity. Here, the experimental results agreed with the computer-based predictions. However, the relatively lower FI value of HBx1, HBx3, and HBx4 indicated that the affinity of these peptides was less than expected. The DC50 assay also showed that HBx2 had the best binding stability (>2 hrs). However, even though the performance of other peptides in T2 binding assay was not ideal, the binding stability of HBx4 was greater than that of HBx2. We suspect that specific residues in the epitopes may be responsible for much of the binding stability. An ideal CTL epitope should be not only antigenic, but also immunogenic (Sable *et al.*, 2007). The antigenicity of peptides can be demonstrated by the binding with HLA molecules. Whether these peptides were immunogenic and could induce CTL was a crucial element of the cell-mediated immune response. We demonstrated the antigenicity of candidate peptides by the analysis of T2-HLA binding. Furthermore, we analyzed the efficacy of four peptides in the activating peptide-specific CTL responses using IFN-γ ELISPOT and LDH release assay. The results showed that all the candidate peptides were able to activate specific T cells responses *in vitro*. HBx2 could induce peptide-specific CTLs not only to secret IFN-γ, but also to kill target cells. The CTL response induced by any other peptide produced only weak IFN-γ secretion and cytotoxicity. This indicated that HBx2 possessed both antigenicity and immunogenicity in accord with the previous studies (Chung *et al.*, 1999; Malmassari *et al.*, 2005).

Some errors in antigen presentation can influence the immunogenicity of peptides. Such errors include a proteasome degradation by mistake, failure to be transported by TAP, and a small number of specific T cell receptors to identify them (Rivett and Hearn, 2004; Gurnani *et al.*, 2004). Therefore, even though the HBx1, HBx3, and HBx4 epitopes showed ideal T2 cells binding or T cell stimulation, the lower immunity responses they induced in our experiments reveal that they are not CTL epitopes with the appropriate immunogenicity. Nevertheless, further research on the physical and chemical characteristics of these ineffectual peptides may improve the efficiency of epitope prediction.

We used a tetramer staining method to analyze the HBV-specific CTL response of the candidate peptides. The tetramer was confirmed to have binding activity that enabled it to recognize HLA-A0201-restricted CTLs in HBV-infected

patients with different disease stages, namely, chronic hepatitis, liver cirrhosis, and hepatoma. The positive rate of tetramer staining in all three stages of disease was greater than 75%. Interestingly, the positive rate in patients with liver cirrhosis was 90% that was higher than in the other patient groups. Tetramer staining can detect functional CTLs with minimal manipulation, so they are more likely to maintain their natural *in vivo* status.

To our knowledge, this is the first report analyzing HBx-specific CTLs in HBV-infected patients using a HLA-A0201-restricted tetramer. Our research indicated that HBx2 in the X antigen of HBV was a potential HLA-A0201-restricted CTL epitope that could be of diagnostic value for the HBV-induced diseases.

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