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• VIRAL HEPATITIS •

Therapeutic polypeptides based on HBV core 18-27 epitope can induce CD_8^+ CTL-mediated cytotoxicity in HLA-$A2^+$ human PBMCs

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Abstract

AIM: To explore how to improve the immunogenicity of HBcAg CTL epitope based polypeptides and to trigger an HBV-specific HLA I-restricted $CD8^+$ T cell response *in vitro*.

METHODS: A new panel of mimetic therapeutic peptides based on the immunodominant B cell epitope of HBV PreS2 18-24 region, the CTL epitope of HBcAg18-27 and the universal T helper epitope of tetanus toxoid (TT) 830-843 was designed using computerized molecular design method and synthesized by Merrifield's solid-phase peptide synthesis. Their immunological properties of stimulating activation and proliferation of lymphocytes, of inducing T_{H1} polarization, $CD8^+$ T cell magnification and HBV-specific $CD8^+$ CTL mediated cytotoxicity were investigated *in vitro* using HLA-$A2^+$ human peripheral blood mononuclear cells (PBMCs) from healthy donors and chronic hepatitis B patients.

RESULTS: Results demonstrated that the therapeutic polypeptides based on immunodominant HBcAg18-27 CTL, PreS2 B- and universal T_H epitopes could stimulate the activation and proliferation of lymphocytes, induce specifically and effectively $CD8^+$ T cell expansion and vigorous HBV-specific CTL-mediated cytotoxicity in human PBMCs.

CONCLUSION: It indicated that the introduction of immunodominant T helper plus B-epitopes with short and flexible linkers could dramatically improve the immunogenicity of short CTL epitopes *in vitro*.

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INTRODUCTION

Despite the existence of effective vaccines against hepatitis B virus (HBV) for many years and massive prophylactic vaccination campaigns, HBV infection remains an important health problem worldwide. HBV can evade the immune defence system and present consistently in hepatocytes. Patients carrying the virus can develop chronic hepatitis, liver cirrhosis, and ultimately hepatocellular carcinoma[1-3]. Currently, the two approved therapies for chronic hepatitis with definite clinical beneficial effects are IFN-α and lamivudine. IFN-α therapy combines antiviral and immunostimulant properties and can result in sustained suppression of HBV replication in one-third of patients. Lamivudine leads to a rapid and almost absolute discontinuation of HBV replication as well as a rapid improvement of the necro-inflammatory activity of the liver disease and to a lesser extent of fibrosis. However, short-term treatment leads to a frequent relapse of HBV replication. On the other hand, long-term treatment has shown to result in virological breakthrough related to the selection of resistant viral variants with a yearly incidence of 15-25%. These outcomes emphasize the need for novel therapeutic approaches[4]. And it is known that cytotoxic T lymphocytes(CTLs) recognize short peptides derived from the intracellular processing of viral antigens in association with HLA class I molecules on the surface of the infected cells, and HLA-I restricted T cell mediated responses, especially virus antigen specific CTL mediated cytotoxicity, play the key role in controlling HBV infection and in the clearance of infected cells[5-7]. Since HBV does not efficiently infect human cells *in vitro*, the use of short synthetic peptides comprised of a set of immunodominant epitopes of virus antigens mimicking the processed antigen fragments can be a rational strategy to stimulate the HBV specific CTL response and break to some extent the immune tolerance to HBV antigens[8-12]. Based on this concept, a new panel of short polypeptides (mimogens) representing the immunodominant CTL, B- and T helper epitopes of the HBcAg, pre-S2 and tetanus toxoid was designed and used for CTL-mediated response analysis. This issue was addressed *in vitro* with HLA-$A2^+$ human peripheral blood lymphocytes (PBMCs) from healthy donors and chronic hepatitis B patients.

MATERIALS AND METHODS

Materials

HLA-$A2^+$ human peripheral blood mononuclear cells (PBMCs) from 9 healthy donors and 9 chronic viral hepatitis B patients were kindly donated by Southwest Hospital, Chongqing municipality, China. Amino acids used for peptide synthesis were purchased from PE & ACT companies. $Na_2{}^{51}GrO_4$ for target cell labeling in standard ^{51}Gr release assay and ^3H-TdR were both from New England Nuclear (NEN™), Boston, USA. Other materials used in this study were as the following: RPMI 1640 medium (Gibco), fetal calf serum (FCS) (HyClone), HLA-A*0201/FLPSDFFPSV tetramer kit (ProImmune, UK) and human IFN-γ ELISpot kit (DiaClone, France).

Methods

Mimetic polypeptides The immunodominant B- and CTL epitopes of HBV pre-S2 and HBcAg were identified on the basis of the HLA-A2.1 binding motifs[13-15]. Peptide1 was chosen from the immunodominant $HBcAg_{18-27}$ CTL epitope (FLPSDFFPSV). The N-termini of peptide 1 linking to the universal T helper sequence of TT 830-843 through a linker of "-Gly-Gly-Gly-" was named as peptide 2 (QYIKANSKFIGITE GGG FLPSDFFPSV). The universal T helper epitope of tetanus

toxoid and the Pre-$S2_{18-24}$ B-epitope were linked to the N- and C-termini of the $HBcAg_{18-27}$ sequence respectively with the linker of"-Ala-Ala-Ala-" and "-Gly-Gly-Gly-" as peptide 3 (QYIKANSKFIGITE AAA FLPSDFFPSV GGG DPRVRGLYFPA). Melanoma associated $MART\text{-}1_{27-35}$ CTL epitope peptide (AAGIGILTV) was used as irrelevant control. All mimogens were calculated and optimized using computerized molecular design theories and methods in O2 workstation(SGI).

The above peptides were synthesized with a Merrifield' s solid-phase peptide synthesis method (PE431A synthesizer), purified by RP-HPLC (WATERS 600) and analyzed by MS/MS (API 2000). All peptides with a purity over 95% were solved in DMSO at the concentration of 10 mg/mL and preserved at -70 ℃.

Lymphocytes proliferation assay

Human PBMCs were separated from peripheral blood by centrifugation on Ficoll-Hypaque gradients and used as fresh samples[16]. PBMCs were plated at a concentration of 2×10^6/mL in 96-well microplates in PRMI 1640 medium supplemented with 100 mL/L fetal bovine serum, 5×10^{-5} mol/L α-mercaptoethanol, and in the presence of 10 μg/mL mimetic peptides respectively. Eighteen to 24 h later, 1 μCi/well of ^{3}H-TdR was added into the medium. Four to 6 h later, lymphocytes were collected and counted using a β-counter. Non-stimulated PBMCs were used as negative control. Results of samples were considered positive if the stimulation index (SI)>2.1.

T_{H1} polarization assay

For the assay of T_{H1} polarization induced by mimetic peptides, IFN-γ ELISPOT kit was used. Briefly[16,17], 96-well PVDF membrane-bottomed plates were coated with capture anti-human IFN-γ mAb at 4 ℃ overnight. Fresh PBMCs were stimulated with 10 μg/mL of mimetic peptide1, 2 and 3 respectively, and then added to triplicated wells at 5×10^3/well and the plates were incubated for 18 h at 37 ℃ in 50 mL/L CO_2. At the end of incubation, cells were washed off and a second biotinylated anti-IFN-γ mAb was added, followed by streptavidin-alkaline phosphatase conjugate and substrates. After the plates were washed with tap water and dried overnight, spots were counted under a stereomicroscope. The number of T_{H1} polarized cells ($HBcAg_{18-27}$-specific $CD8^+$ T cells), expressed as IFN-γ secreting cells(ISCs) /10^6 PBMCs, was calculated after subtracting negative control values (non-stimulated PBMCs). Results of samples were considered as positive if above the mean by three standard deviations and with a cut off of 50 ISCs /10^6 PBMCs above mean background.

Cytotoxicity assay

Peptide-specific CTL lines were primed as follows: at d 0, fresh PBMCs were plated at a concentration of 2×10^5/mL in 24-well microplates (2 mL /well) in RPMI 1640 medium supplemented with 100 mL/L fetal bovine serum and L-glutamine, and in the presence of 10 μg /mL mimetic peptides respectively. Two days later, 30 IU/mL rhIL-2 was added to the medium. Lymphocytes were then re-stimulated weekly for 2 wk as follows: Cells were harvested, washed once, and replated in 24-well plates at the concentration of 2×10^5/mL in the above medium, and restimulated respectively with 10 μg/mL mimetic polypeptides. Twenty hours after last stimulation, cells were harvested, and used as fresh effectors.

CTL-mediated cytotoxicity was detected by standard 4 h ^{51}Cr release assay[17]. T2(HLA-A2) cells were used as targets and pre-incubated with 10 μg/mL of $HBcAg_{18-27}$ peptide 2 h before use. The 1×10^6 target cells were labeled with 3.7×10^6 Bq $Na_2{}^{51}GrO_4$ in 1.0 mL RPMI 1640 medium supplemented with 150 mL/L fetal bovine serum and in the presence of 10 μg/mL of $HBcAg_{18-27}$ peptide for 60 min at 37 ℃, and then washed three times before the addition of effectors. Various concentrations of effector cells were mixed with 1×10^4 targets at effector/target (E/T) ratios of 12.5, 25, 50 and 100 in 200 μL of culture medium in 96-well V-bottomed microplate in triplicate. The microplate was centrifuged for 3 min at 500 r/min, and then incubated for 4 h at 37 ℃ in 50 mL/L CO_2. After the incubation terminated, 100 μL of supernatants was harvested and counted on a γ-counter. Percentage of target cell specific lysis was determined as:(average sample counts-average spontaneous counts/average maximum counts-average spontaneous counts) ×100%. Maximum and spontaneous counts were measured using supernatants from wells receiving 50 g/L SDS or culture medium alone, respectively. In all experiments, spontaneous counts should be less than 30% of maximum counts. CTL responses were considered positive if they exceeded the mean of specific lysis caused by irrelevant mimetic antigen by three standard deviations and by 10%.

$HBcAg_{18-27}$-specific $CD8^+$ CTL quantitative detection

HLA class I^{pep} tetramer-binding assay was used to quantify the $HBcAg_{18-27}$-specific $CD8^+$ T cells from the fresh effectors produced[18]. Briefly, fresh effectors were collected, washed twice with 0.02 mol/L, pH7.2 phosphate buffered saline (PBS), counted and separated equally into different tubes in 1.0 mL of PBS each. The effectors were stained with 2 μL of R-PE-conjugated HLA-A*0201/FLPSDFFPSV and 20 μL of Cy-Chrome-conjugated mouse anti-human CD8 mAb for 30 min at room temperature. R-PE-conjugated avidin and Cy-Chrome-conjugated mouse $IgG_{1,\kappa}$ antibodies were used as isotype control, and non-stimulated PBMCs were used as negative controls. All samples were collected, washed twice, dissolved into 300 μL of PBS and FACS-sorted on a FACstar (Beckton-Dicknson) with Cell Quest software. Results were expressed as percentages of tetramer-binding cells in the $CD8^+$ population. A total of 50 000 events were acquired in each analysis. Results were considered as positive for tetramer-binding cells when above negative controls and by 0.1% $CD8^+$ T cells.

Statistical analysis

All data were expressed as mean±SD. Statistical analysis was performed using a two-tailed Student' s *t* test.

RESULTS

Lymphocytes proliferation assay

Fresh PBMCs were stimulated respectively with three mimetic peptides we designed, and ^{3}H-TdR was used to detect the proliferation of lymphocytes. Data demonstrated that peptide 3 pulsed the most vigorous activation and proliferation of lymphocytes, and with SI>4.1 by average in healthy PBMCs and >3.3 in PBMCs from chronic hepatitis patients. Peptide 2 could also induce weak lymphocytes proliferation, with the mean of SI>2.3 and 2.1 respectively in the PBMCs from healthy donors and chronic hepatitis patients. No activation and proliferation of lymphocytes were detected in peptide 1 stimulated PBMCs (Table 1).

T_{H1} polarization induced by mimetic peptides

$HLA\text{-}A2^+$ human PBMCs were pulsed respectively with three mimetic peptides we initially designed and synthesized, and the T_{H1} polarization induced was detected using IFN-γ ELISPOT method. Spots of IFN-γ secreting cells can be observed in each of the mimetic peptide samples. Peptide1 could induce peptide-specific $CD8^+$ T cells magnification up to approximately 1 667±231 ISCs /10^6 PBMCs in PBMCs from healthy donors and 1 420±253 ISCs /10^6 PBMCs in PBMCs from chronic hepatitis B patients, which were dramatically weaker than those of Peptide 2 and 3, which induced

Table 1 Lymphocytes proliferation assay (mean±SD, n=27)

	^{3}H-TdR counts(*cpm*)			
	Peptide1	Peptide2	Peptide3[b]	Negative controls
PBMCs from healthy donors	4 001.6±328.3	5 882.6±397.2	10 497.6±859.7	2 556.3±211.3
PBMCs from chronic hepatitis patients	3 967.5±285.9	4 912.3±421.1	7 696.2±781.8	2 327.6±169.2

[b]P<0.01 *vs* negative control and peptide1 and 2 groups.

Table 2 Peptide-specific CD8$^+$ T cells induced expressed as ISCs/10^6 PBMCs (mean±SD, n=27)

	ISCs /10^6 PBMCs				
	Peptide1[d]	Peptide2[d]	Peptide3[db]	Irrelevant control	Negative controls
PBMCs from healthy donors	1 667.5±231.3	4 133.7±416.6	9 200.5±1638.1	1 315.5±321.3	233.3±208.6
PBMCs from chronic hepatitis patients	1 420.0±253.5	3 915.7±685.9	8 966.7±1435.3	1 230.0±355.2	245.1±223.3

[b]P<0.01 *vs* peptide1 and 2 groups, [d]P<0.01 *vs* negative control.

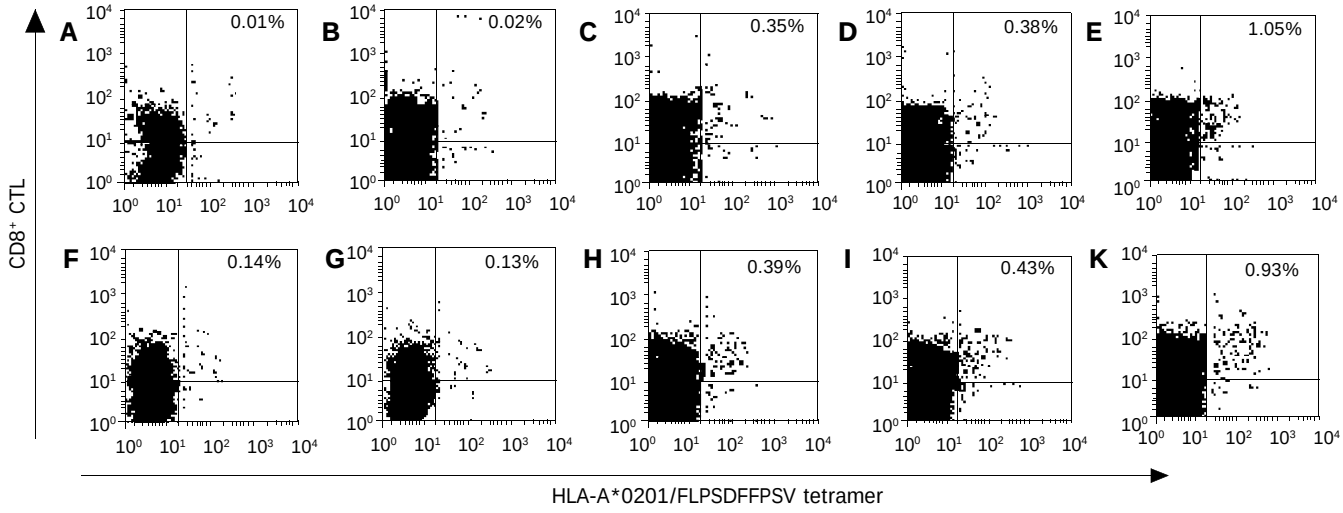

Figure 1 Detection of the HBcAg$_{18\text{-}27}$-specific CD8$^+$ T cells produced with HLA-A*0201/FLPSDFFPSV tetramer-binding assay. A: Non-stimulated HLA-A2$^+$ PBMCs from healthy donors; B: Irrelevant peptide pulsed HLA-A2$^+$ PBMCs from healthy donors; C: Peptide1 pulsed HLA-A2$^+$ PBMCs from healthy donors; D: Peptide2 pulsed HLA-A2$^+$ PBMCs from healthy donors; E: Peptide3 pulsed HLA-A2$^+$ PBMCs from healthy donors; F: Non-stimulated HLA-A2$^+$ PBMCs from chronic hepatitis patients; G: Irrelevant peptide pulsed HLA-A2$^+$ PBMCs from chronic hepatitis patients; H: Peptide1 pulsed HLA-A2$^+$ PBMCs from chronic hepatitis patients; I: Peptide2 pulsed HLA-A2$^+$ PBMCs from chronic hepatitis patients; K: Peptide3 pulsed HLA-A2$^+$ PBMCs from chronic hepatitis patients.

approximately 4 133±416 and 9 200±1 638 ISCs/10^6 PBMCs respectively in PBMCs from healthy donors, and 3 915±685 and 8 966±1 435 ISCs/10^6 PBMCs in PBMCs from chronic hepatitis B patients. These results suggest that all the 3 peptides could pulse T_{H1} polarization of T cells, and peptide 3 was more vigorous than peptide 2 and 1(P<0.01). It showed no difference between the PBMCs from healthy donors and chronic hepatitis patients (Table 2).

Cytotoxicity assay

HLA-A2$^+$ human PBMCs were pulsed with the 3 mimetic peptides and the irrelevant control peptide respectively, and the CTL-mediated cytotoxicity induced was tested by standard 4 h ^{51}Cr release assay against HBcAg$_{18\text{-}27}$ peptide pre-incubated T2 targets. The results demonstrated that all the 3 mimetic peptides could induce positive HBV-specific CTL response, among which peptide 3 induced the most vigorous CTL activity and as high as (68.4±15)% target cell lysis was observed at E/T=100. The percentages of target cells lysed between peptide 1 and 2 pulsing groups were of statistically no difference, and both were dramatically lower than that of peptide 3 (P<0.01). The targets lysis observed in both healthy donors and chronic hepatitis patients showed no statistical difference (Tables 3 and 4).

HBcAg$_{18\text{-}27}$-specific CD8$^+$ CTL detection

HLA-A2$^+$ human PBMCs were pulsed respectively with the above three mimetic peptides and the irrelevant peptide MART-1$_{27\text{-}39}$, the HBcAg$_{18\text{-}27}$-specific CD8$^+$ T cells induced were quantified using HLA-A*0201/FLPSDFFPSV tetramer-binding assay. No HBcAg$_{18\text{-}27}$-specific CD8$^+$ T cells could be detected in the PBMCs pulsed with MART-1$_{27\text{-}35}$ peptide, and the tetramer staining was almost the same as control background (0.02% in PBMCs from healthy donors, and 0.04-0.14% in PBMCs from chronic hepatitis patients). In PBMCs stimulated with peptide 1, 2 and 3, the frequencies of HLA-A*0201/FLPSDFFPSV· CD8 double-positive T cells were on average 0.35%(3 500/10^6 PBMCs), 0.38%(3 800/10^6 PBMCs) and 1.05% (10 500/10^6 PBMCs) respectively in the PBMCs from healthy donors, and 0.39%(3 900/10^6 PBMCs), 0.43%(4 300/10^6 PBMCs) and 0.93%(9 300/10^6 PBMCs) respectively in the PBMCs from chronic patients. Data showed no statistical differences between the PBMCs from healthy donors and chronic hepatitis patients, and between the effects induced by peptide1 and peptide 2 (Figure 1).

Table 3 Percentages of specific targets lysis by HLA-A2$^+$ effector CTLs induced by PBMCs from healthy donors with different peptide antigens (mean±SD, n=27)

E/T ratios	Percentage of specific cell lysis (%)				
	Peptide1[d]	Peptide2[d]	Peptide3[db]	Irrelevant control[a]	Negative controls
12.5	22.7±5.3	21.7±6.1	36.1±7.7	3.7±0.7	3.5±0.7
25	29.3±6.5	28.6±6.3	41.4±9.1	5.7±0.7	3.7±0.7
50	33.5±7.1	33.9±8.2	52.3±12.5	7.3±0.9	6.4±0.9
100	37.2±11.2	36.8±10.9	68.4±14.7	7.4±1.1	8.7±1.3

[a]P>0.05 *vs* negative control, [b]P<0.01 *vs* peptide1 and 2 groups, [d]P<0.01 *vs* negative control.

Table 4 Percentages of specific targets lysis by HLA-A2$^+$ effector CTLs induced by PBMCs from chronic hepatitis B patients with different peptide antigens (mean±SD, n=27)

E/T ratios	Percentage of specific cell lysis (%)				
	Peptide1[d]	Peptide2[d]	Peptide3[db]	Irrelevant control[a]	Negative controls
12.5	18.7±3.6	23.1±4.9	30.2±6.1	5.1±0.8	4.3±0.6
25	22.7±4.1	28.6±5.6	38.4±7.4	4.5±0.7	7.1±1.0
50	31.3±6.0	33.9±6.5	52.7±10.3	7.3±1.1	7.7±1.3
100	35.5±5.7	39.1±7.3	58.5±15.9	9.5±1.7	8.5±1.5

[a]P>0.05 *vs* negative control, [b]P<0.01 *vs* peptide1 and 2 groups, [d]P<0.01 *vs* negative control.

DISCUSSION

HBV-specific CD8$^+$ cytotoxic T cells play a critical role in viral clearance. Low HBV-specific CTL responses in chronic HBV infection may favor the persistence of virus, whereas stimulation and expansion of HBV-specific CTL activity may assist elimination of HBV infection[1-3]. Natural HBV antigens generally contain inappropriate epitopes which could elicit T_{H1}/T_{H2} disequilibrium, immune deviation or immune deficiency, and the conserved amino acid sequences might interfere with intercellular communication and thus elicit immune subversion. Thereby some viruses may evade the immune defence system and present consistently in hepatocytes, and result in chronic hepatitis, liver cirrhosis, and even hepatocellular carcinoma. Thus new generations of therapeutic vaccines should induce CTL responses different from that induced by natural virus infection, and at the same time hold the specificity of HBV antigens[19-22]. According to modern immune theories, effective protection relys on the appropriate match of a set of epitopes[8,23]. Thus, natural antigens should be redesigned or modified using molecular design techniques on the basis of immunodominant epitopes.

Among the different CTL epitopes of HBV core, envelope, and polymerase so far identified, the sequence 18-27 of the HBV core antigen is immunodominant and subdominant in the different supertype of HLA-A2 molecules, and could induce HBV-specific CTL responses in patients of different HLA-A2 subertypes with indistinguishable frequency and magnitude, and represents the main component of a peptide-based therapeutic vaccine aiming at stimulating the antiviral CTL response in patients with chronic hepatitis B. Furthermore, this epitope was also found to stimulate HLA class II restricted T-cell responses. These data illustrate its potential usefulness for the development of therapeutic vaccines[24-26].

As in other infections with noncytopathic viruses, helper T cells control the intensity of CD8$^+$ T-cell responses and helper T-cell responses might be compromised in chronic carriers of HBV, and according to *in vivo* studies, administration of single CTL epitope vaccine could initiate CTL activity, but the magnitude was lower, and the low-level CTL activity was considered not associated with viral clearance[26-30]. In this study, we chose the immunodominant B cell epitope of HBV $PreS_2$ region and the CTL epitope of HBcAg, and introduced the universal T_H epitope of tetanus toxoid to strengthen the T_H response. Three mimetic peptides based on the above epitopes were initially designed and synthesized, and their immunological properties of pulsing lymphocyte activation and proliferation, of inducing T_{H1} polarization and HBV-specific CD8$^+$ CTL-mediated cytotoxicity were preliminarily investigated using human PBMCs from HLA-A2$^+$ healthy donors and chronic hepatitis B patients.

After *in vitro* stimulation, a direct tetramer-binding assay was used to detect the frequencies of HBV-specific CD8$^+$ T cells. The results varied according to the peptides used. The highest frequencies were from peptide3 pulsing group, about 1.05%(10 500/10^6 PBMCs) and 0.93%(9 300/10^6 PBMCs) HLA-A*0201/$HbcAg_{18-27}$· CD8$^+$ CTLs produced in the PBMCs from healthy donors and chronic hepatitis patients respectively, remarkably higher than those of peptide 2 and peptide 1(P<0.01). No $HBcAg_{18-27}$-specific CD8$^+$ T cells could be detected in the PBMCs pulsed with irrelevant peptide, the tetramer staining was almost the same as control background. These data demonstrated the specificity of the therapeutic peptides we designed.

The tetramer-binding assay detects only the number of cells with an appropriate TCR but not their function[31], so chromium release assay, IFN-γ ELISpot assay and lymphocyte proliferation assay were used to detect the function of the effectors pulsed. And a highly significant correlation was found between the frequencies of peptide-specific CD8$^+$ T cells and the functions of responding T cells. All the three mimetic polypeptides designed were potent to induce *in vitro* cultured human PBMCs activation and proliferation, T_{H1} polarization, CD8$^+$ T cell expansion and generation of cytotoxicity. Peptide 3 with the immunodominant B-, CTL and T helper epitope was the most potent. After introduction of T helper epitope into peptide 1, CTL frequency was not remarkably improved, and cytotoxic activity remained low suggesting that this conformation was not sufficient to drive proliferation of CTLs, and its differentiation into mature killer cells. The comparatively higher immunogenicity of peptide 3 was attibutedd to its molecular structure: the introduction of T helper and B-epitopes, and the design of short linkers "-A-A-A-" and "-G-G-G-". The linker was designed and proved to be highly flexible and might act as "hinges". We surmise that this peptide might be recognized by MHC-I/II restricted molecules, and be presented to CD4$^+$ T cells and CD8$^+$ T cells, and ultimately T helper and Tc cells should be activated and function interactively. The results demonstrate that the peptides designed are highly immunogenic and HBV-specific, and the introduction of short and flexible linker and immunodominant T_H plus B- epitopes into short CTL epitopes may dramatically improve the therapeutic peptides' immunogenicity and the possibility of being presented to antigen presenting cells (APCs).

According to reports as yet, the vast majority of polypeptides, especially short epitope peptides can not induce CTL responses vigorous by *in vivo* because of poor immunogenicity[32-35]. Little knowledge is known so far on the molecular mechanisms leading to the difference between the peptides' *in vitro* and *in vivo* functions. In our opinion, *in vivo* induction of cytotoxic activity relys on the efficient presentation by APCs, and the crucial point is how to improve the antigenicity of short peptides and to meet the needs for efficient antigen presentation *in vivo*. In the present study, we redesigned and modified the structure of linear short peptides on the basis of immunodominant

epitopes, changed the molecular properties of the natural peptides, and triggered the direct recognition of the peptides by Tc and T_H cells, and the mimogens sieved were proved to be highly immunogenic *in vitro*. Whether this conformation can meet the needs for efficient antigen presentation *in vivo* needs to be addressed in HLA-A2 transformed HBV transgenic mice.

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