Therapeutic polypeptides based on HBcAg<sub>18-27</sub> CTL epitope can induce antigen-specific CD<sub>8</sub><sup>+</sup> CTL-mediated cytotoxicity in HLA-A2 transgenic mice

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ABSTRACT

AIM: To explore how to trigger an HLA-I-restricted CD8<sup>+</sup> T cell response to exogenously synthesized polypeptides in vivo.

METHODS: Three mimetic therapeutic polypeptides based on the immunodominant CTL epitope of HBcAg, the B- epitope of HBV PreS<sub>2</sub> region and a common T helper epitope showed an appropriate match of the above CTL, B- and T helper epitopes in HLA-A2 transgenic mice. The mimetic polypeptides could trigger specifically and effectively vigorous CD8<sup>+</sup> HBV-specific CTL-mediated cytotoxicity and Th<sub>1</sub> polarization of T cells in HLA-A2 transgenic mice.

RESULTS: Results demonstrated that the mimetic polypeptides comprised of the immunodominant CTL, B-, and T helper epitopes could trigger specifically and effectively vigorous CD8<sup>+</sup> HBV-specific CTL-mediated cytotoxicity and Th<sub>1</sub> polarization of T cells in HLA-A2 transgenic mice.

CONCLUSION: A designed universal T helper plus B-epitopes with short and flexible linkers could dramatically improve the immunogenicity of CTL epitopes in vivo. And that the mimetic therapeutic peptides based on the reasonable match of the above CTL, B- and T helper epitopes could be a promising therapeutic peptide vaccine candidate against HBV infection.

INTRODUCTION

At present, the commercial vaccines against HBV infection are mainly based on humoral responses which can prevent the virus infection, but can rarely interrupt the intracellular infection or lead to the infected cell clearance. As in other infections with noncytopathic viruses, an MHC class I-restricted cytotoxic T lymphocytes (CTLs) response to endogenous HBV antigens is believed to be the major determinant for infected cell clearance, and HBV-specific CTL-mediated cytotoxicity plays the key role in controlling HBV infection and in the clearance of infected cells.[1-8]

Natural HBV antigens contain generally inappropriate epitopes which could elicit T<sub>M</sub>/T<sub>H</sub> disequilibrium, immune deviation or immune deficiency, and the conserved amino acid sequences might also interfere with intercellular communication. Therefore some viruses may evade the immune defence and present consistently in hepatocytes, and result in chronic hepatitis, liver cirrhosis, and even hepatocellular carcinoma.[9-19]. 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. According to reports in recent years, effective protection relies on the appropriate match of a set of epitopes[9,20]. Thus, natural antigens should be redesigned or modified on the basis of immunodominant epitopes.

In this study, we chose the immunodominant CTL epitope of HBcAg and the B cell epitope of HBV PreS<sub>2</sub> region, and introduced them into the common T helper epitope of tetanus toxoid to strengthen the Th response. Three mimetic peptides based on the above epitopes were initially designed and synthesized, and their immunological functions of inducing Th<sub>1</sub> polarization, CD8<sup>+</sup> HBV-specific CTL expansion and CD8<sup>+</sup> HBV-specific CTL-mediated cytotoxicity were investigated in HLA-A2 transgenic mice. We aimed to explore how to trigger an HLA-I-restricted CD8<sup>+</sup> T cell response to exogenously synthesized polypeptides in vivo, and to find a rational strategy of stimulating the HBV specific CTL response in vivo and to break to some extent the immune tolerance to HBV antigens.

MATERIALS AND METHODS

Materials

Inbred male and female HLA-A2 transgenic mice (H-2K<sup>b</sup>) aging 6-8 wk were purchased from Jackson Laboratory, USA. Amino acids used for peptide synthesis were purchased from PE & ACT companies. Na<sub>2</sub>CrO<sub>4</sub> for target cell labeling in standard 51Cr release assay was from New England Nuclear (NEN<sup>TM</sup>), Boston, USA. There were also materials used in this study as the following: RPMI1640 medium (Gibco), fetal calf serum (FCS) (HyClone), chimeric HLA-A2K<sup>b</sup> tetramer kit (ProImmune, UK) and murine IFN-γ ELISPOT kit (Diaclone, France).

Methods

Preparation of mimetic therapeutic polypeptides

The immunodominant B- and CTL epitopes of HBV pre-S2 and HBcAg were identified on the basis of the HLA-A2.1 binding motif[21]. Mimetic polypeptides were calculated and sieved using computerized molecular design methods. Peptide1 was determined as the immunodominant HBcAg<sub>18-27</sub> CTL epitope peptide (FLPSDFFPSV), to the N-termini of which linked the common T helper sequence of tetanus toxoid with a linker of “-Gly-Gly-Gly-” as peptide2 (QYIKANSKFIGITE GGG FLPSDFFPSV). The common T helper epitope of tetanus
toxoid and the Pre-S2 B-epitope were linked to the N- and C-termini of the HBcAg18-27 sequence respectively with the linker of ‘-Ala-Ala-Ala-‘ and ‘-Gly-Gly-Gly-‘ as peptide 3 (QYIKANSFIGHTAAALPSPDFSSTVGGDGPRVGLYYPFA). Melanoma associated MART-127-35 CTL epitope peptide (AAGGIGILTV) was used as irrelevant control.

The above peptide antigens were synthesized with the 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 dissolved in DMSO with the concentration of 10 mg/mL and preserved at -70 °C.

**Immunization of mice** Mimetic polypeptides in DMSO were diluted with 0.02 mol/L, pH 7.2 phosphate buffered saline (PBS) and emulsified respectively with complete Freund’s adjuvants (CFA) and incomplete Freund’s adjuvants (IFA). HLA-A2 transgenic mice were separated into 5 groups with 3 groups in each. The mice were injected subcutaneously in the 2 flanks and in the hind footpads first with peptide1, 2, 3 and then irrelevant control peptide in CFA at 500 µg/kg dosage respectively. Two weeks later, the mice were immunized weekly with the same antigens at IFA in 250 µg/kg dosage for 4 times. The mice injected with PBS alone were used as negative controls.

Two weeks after the last time of immunization, the mice were killed, the splenocytes were separated and suspended in RPMI1640 medium supplemented with 100 µL/L FCS and used as fresh samples.

**Tₜh polarization assay** For the assay of Tₜh polarization induced by mimetic peptides, mouse IFN-γ ELISPOT kit was used. Briefly[19,22] 96-well PVDF membrane-bottomed plates were coated with capture anti-mouse IFN-γ mAb at 4 °C overnight. Fresh splenocytes were added to triplicated wells at 5x10³/well in the presence of 10 µg/mL relative mimetic antigens and the plates were incubated for 15 h at 37 °C in 50 µL/L CO₂. 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 Tₜh polarized cells (peptide-specific CD8⁺ T cells), expressed as IFN-γ secreting cells (ISC)/10⁶ lymphocytes, was calculated after subtracting negative control values. Results of samples were considered as positive if above the mean by three standard deviations and with a cut off of 50 ISC/10⁶ lymphocytes above mean background.

**Cytotoxicity assay** Peptide-specific CTL lines were primed as follows: The fresh splenocytes were plated at a concentration of 2x10⁶/mL in 24-well microplates (2 mL/well) in RPMI1640 medium supplemented with 100 µL/L FCS and 30 U/mL murine IL-2 and in the presence of 10 µg/mL corresponding mimetic peptides respectively, with an exception of the lymphocytes from negative controls which were just cultivated in medium supplemented with 100 µL/L FCS and 30 U/mL murine IL-2. Five days after stimulation, cells were harvested, and used as fresh effectors.

**Cytotoxicity assay** following peptide antigen stimulation was detected by a standard 4 h ⁵¹Cr release assay[18,19,23,24]. P815 cells were used as targets and preincubated with HBcAg18-27 peptide 2 h before use. The 1x10⁶ target cells were labeled with 3.7x10⁶ Bq Na⁵¹CrO₄ in 0.8 mL RPMI1640 medium supplemented with 150 µL/L FCS for 60 min at 37 °C, and then washed 3 times before the addition of effectors. Various concentrations of effector cells were mixed with 1x10⁴ 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 triplicates. The microplate was centrifuged for 3 min at 500 r/min, and then incubated for 4 h at 37 °C in 50 µL/L CO₂. After the incubation terminated, 100 µL/well 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)] x100%. Maximum and spontaneous counts were measured using supernatants from wells receiving 1 mol/L HCl or culture medium alone, respectively. In all experiments, spontaneous counts should be less than 30% of maximum counts. CTL responses were considered positive if exceeded the mean of specific lysis caused by irrelevant mimetic antigen (MART-127-35) by 3 standard deviations and by 10%.

**HBCAg18-27 specific CD8⁺ CTL detection**

Chimeric HLA-A2K tetramer binding kit was used to quantify the HBcAg18-27-specific CD8⁺ T cells[22-24]. A part of the fresh splenocytes separated from mice killed were washed with PBS, counted, suspended in PBS and separated equally into different tubes in 1.0 mL of each. The cells were stained with 25 ng/µL of tetramers for 20 min at 37 °C, and then washed and stained with anti-mouse CD8 mAb for 20 min at room temperature. All samples were collected, washed twice, dissolved into 300 µL of PBS and FACS-sorted on a FACstar (Beckton-Dickinson) with Cell Quest software. Results were expressed as percentages of tetramer-binding cells in the murine CD8⁺ T cell population. A total of 5x10⁶ events were acquired in each analysis. Results were considered as positive for tetramer-binding cells when above the mean caused by irrelevant mimetic antigen (MART-127-35) and above 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**

**Tₜh polarization induced by mimetic peptides**

When the immunization terminated, the mice were killed and the spleen lymphocytes were used for Tₜh polarization assay with an IFN-γ ELISPOT method. Spots of IFN-γ secreting cells generated could be observed in each of the mimetic peptides immunized groups. The negative control values of peptide-specific CD8⁺ T cells were on average 250±138 ISC/10⁶ lymphocytes. The most vigorous peptide-specific CD8⁺ T cells magnification was produced in peptide3 immunized mice with approximately 8 050±2 233.8 ISC/10⁶ lymphocytes generated. In peptide1 and 2 immunization groups, the peptide-specific CD8⁺ T cells induced were 2 100±236.7 and 1 367±231 ISC/10⁶ lymphocytes respectively. And also 1 135±312 ISC/10⁶ lymphocytes were generated in MART-127-35 CTL epitope peptide immunized mice (Table 1).

**Cytotoxicity assay**

When the mice were killed, the fresh splenocytes were separated and peptide-specific CTL lines were generated ex vivo. The CTL-mediated cytotoxicity induced was tested by standard 4 h ⁵¹Cr release assay against HBcAg18-27 Peptide preincubated P815 targets. Data demonstrated that all the three mimetic polypeptides based on HBcAg18-27 CTL epitope could induce positive HBV-specific CTL response, among which peptide3 induced the most vigorous CTL activity, and as high as (55.3±10.1)% targets lysis was observed at E/T=100. The percentages of targets lysed in peptide1 and 2 immunization groups were dramatically lower than in peptide 3 group (P<0.01) and showed statistically no difference between. The spleen lymphocytes from irrelevant peptide control and negative control mice showed no specific target cell lysis activity (Table 2).
Table 1  T<sub>11</sub> polarization assay with an IFN-γ ELISPOT method. (mean±SD, n=18)

|                  | Peptide1 | Peptide2 | Peptide3 | MART-1<sub>27</sub><sub>35</sub> | Negative controls |
|------------------|----------|----------|----------|-------------------------------|-------------------|
| ISC/10<sup>6</sup> lymphocytes | 1.150±236.7<sup>d</sup> | 1.367±231<sup>d</sup> | 8.050±1 233.8<sup>b</sup> | 1.135±312         | 250±138           |

<sup>d</sup>P <0.01 vs negative control; <sup>b</sup>P <0.01 vs peptide1 and 2 groups.

Table 2  Percentages of specific targets lysis by effector CTLs induced with different peptide antigens (mean±SD, n=18)

| E/T ratios | Percentage of specific cell lysis (%) |
|------------|--------------------------------------|
|            | Peptide1<sup>a</sup> | Peptide2 | Peptide3 | MART-1<sub>27</sub><sub>35</sub> | Negative controls |
| 12.5       | 18.5±4.3<sup>d</sup> | 21.1±6.3<sup>d</sup> | 33.7±7.2<sup>b</sup> | 3.7±0.7         | 5.5±0.9           |
| 25         | 22.9±5.7<sup>d</sup> | 28.5±6.6<sup>d</sup> | 40.5±8.9<sup>b</sup> | 6.3±0.9         | 4.4±0.9           |
| 50         | 29.3±7.0<sup>d</sup> | 30.5±8.8<sup>d</sup> | 50.3±11.3<sup>b</sup> | 7.1±1.1         | 7.7±1.2           |
| 100        | 32.6±10.1<sup>d</sup> | 33.1±8.2<sup>d</sup> | 55.3±10.1<sup>b</sup> | 8.4±1.3         | 9.2±1.3           |

<sup>d</sup>P <0.01 vs negative control; <sup>b</sup>P <0.01 vs peptide1 and 2 groups.

Figure 1  Detection of the HBcAg<sub>27-35</sub>-specific CD8<sup>+</sup> T cells produced with chimeric A2K<sup>+</sup>/HBcAg<sub>27-35</sub> tetramer-binding assay. A: murine splenocytes from negative control mice, B: murine splenocytes from MART-1<sub>27-35</sub> irrelevant peptide immunized mice, C: murine splenocytes from peptide1 immunized mice, D: murine splenocytes from peptide2 immunized mice, E: murine splenocytes from peptide3 immunized mice.

HBcAg<sub>27-35</sub>-specific CD8<sup>+</sup> CTL detection

After the immunization terminated, the HBcAg<sub>27-35</sub>-specific CD8<sup>+</sup> T cells induced in vivo were quantified using chimeric A2K<sup>+</sup>/HBcAg<sub>27-35</sub> tetramer-binding assay. No HBcAg<sub>27-35</sub>-positive CD8<sup>+</sup> T cells could be detected in the spleen lymphocytes from the negative control mice and the mice immunized with MART-1<sub>27-35</sub> peptide, and the tetramer staining was lower than background (0.02%). In splenocytes from the mice immunized with peptide 1, 2 and 3, the frequencies of A2K<sup>+</sup>/HBcAg<sub>27-35</sub> CD8<sup>+</sup> positive T cells were respectively 0.36% (3 600/10<sup>6</sup> lymphocytes), 0.39% (3 900/10<sup>6</sup> lymphocytes), and 0.01% (10 100/10<sup>6</sup> lymphocytes). It showed no statistical difference between the effects induced by peptide 1 and peptide 2, and the immunogenicity of them was dramatically weaker than that of peptide 3 (Figure 1).

DISCUSSION

HBV-specific CD8<sup>+</sup> 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[15,16]. Natural HBV antigens contain some inappropriate epitopes and conserved amino acid sequences which might induce inappropriate immune responses and result in hepatic pathology and lesions. Hence new generations of therapeutic vaccines should be designed on the basis of immunodominant epitopes which could induce CTL responses different from that induced by natural virus infection, and at the same time hold the specificity of HBV antigens. As in other infections with noncytopathic viruses, helper T cells control the intensity of CD8<sup>+</sup> T-cell responses and helper T-cell responses might be compromised in chronic carriers of HBV[27-32]. In this paper, we chose the immunodominant B cell epitope of HBV PreS<sub>1</sub> region and the CTL epitope of HBcAg, and introduced them into the common T helper epitope of tetanus toxoid to strengthen the Th response. Three mimetic peptides based on the above epitopes were initially designed and synthesized, and their immunological properties of inducing T<sub>11</sub> polarization, CD8<sup>+</sup> T-cell expansion and CTL-mediated cytotoxicity were primarily investigated in HLA-A2 transgenic mice.
After immunization, the mice were killed and the splenocytes were separated, a direct tetramer-binding assay was used to detect the frequencies of HBcAg<sub>18-27</sub>-specific CD8<sup>+</sup> T cells, the results varied according to the peptides used. The highest frequency was from peptide 3 immunized mice splenocytes. No statistical difference was observed between the frequencies of HBcAg<sub>18-27</sub>-specific CD8<sup>+</sup> T cells augmented in peptide 1 and peptide 2 mice groups, which indicated that the immunogenicity of short CTL epitope peptides in vivo could not be improved dramatically simply by introduction of a universal T helper epitope, and that by our reasonable match of the above immunodominant CTL. T helper epitopes and linkers, the immunogenicity of HBcAg<sub>18-27</sub> CTL epitope of inducing HBV-specific CD8<sup>+</sup> T cell expansion in vivo was dramatically improved.

The tetramer-binding assay detects only the number of T cells with an appropriate TCR but not their function<sup>24,30</sup>, so a chronicle release assay and IFN-γ ELISPOT assay were used to detect the immune functions of the mimetic antigens designed. And a highly significant correlation was found between the frequencies of HBcAg<sub>18-27</sub>-specific CD8<sup>+</sup> T cells and the functions of responding splenocytes from the mice. The three mimetic polypeptides designed could induce Th<sub>1</sub> polarization of spleen lymphocytes and generate cytotoxicity in mice, among which peptide 3 with the immunodominant B-, CTL and T helper epitopes was the most potent, peptide 1 and 2 could also produce the above immune functions but not as efficiently. After introducing T helper epitope into HBcAg<sub>18-27</sub>, the CD8<sup>+</sup> CTL frequency was not remarkably improved, and cytotoxic activity remained low, suggesting that the help provided by this condition was not sufficient to drive proliferation of CTL, and their differentiation into mature killer cells. The comparatively higher immunogenicity of peptide 3 might rely on its molecular structure: the introduction of T helper and B-epitopes, the design of short linkers “Ala-Ala-Ala” and “Gly-Gly-Gly”, and the reasonable match. The designed linker was proved to be highly flexible and might act as “hinges”. We surmise that the peptide be recognized by MHC-I and II restricted molecules, and be presented to CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells, and ultimately T helper and Tc cells be activated and functioned interactively. It indicated that introducing short and flexible “hinges” and “Th+B” epitopes into short CTL epitope peptides might dramatically improve the peptide’s immunogenicity and the ability of being presented to APCs in vivo. The results also demonstrated that designed peptide 3 was highly immunogenic and HBV-specific in vivo, and might be a potential candidate for the therapeutic vaccine designed against hepatitis B.

Little knowledge is known so far on the molecular mechanisms of the in vitro and in vivo functions of the peptides<sup>8,9,13,30</sup>. In our opinion, in vivo induction of cytotoxic activity relies on the efficient presentation by APCs, and the crucial point is how to improve the antigenicity of short peptides so to meet the needs for efficient antigen presentation in vivo. Thus to redesign or modify the linear short peptides on the basis of immunodominant epitopes, change their molecular properties to meet the needs of antigen presentation, and stimulate the direct recognition of the peptides by T<sub>h</sub>Tc cells may be a promising approach to this problem.

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