A novel HBV DNA vaccine based on T cell epitopes and its potential therapeutic effect in HBV transgenic mice

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Abstract

DNA vaccination represents a novel therapeutic strategy for chronic hepatitis B virus (HBV) infection. Recently, some HBV DNA vaccines have been used in the preliminary clinical trials and exhibited exciting results in chronic HBV carriers. But these vaccines only encoded the single viral antigen, the S or the PreS2/S antigen. In this study, we designed a polytope DNA vaccine encoding multiple T cell epitopes. We found that it induced stronger CTL responses than the vaccine encoding the single antigen in H-2d and H-2b mice, although the CTL response to Ld-restricted epitope suppressed the CTLs to other epitopes in H-2d-restricted mice. Interestingly, heat shock protein 70 as an adjuvant not only enhanced CTL response to the viral antigen but also overcame this epitope suppression. Furthermore, the polytope DNA vaccine resulted in a long-term down-regulation of hepatitis B virus surface antigen and inhibition of HBV DNA replication in a HBV transgenic mouse model. Therefore, our research indicates that it is practicable and feasible to design a polytope DNA vaccine for chronic hepatitis B immunotherapy.

Introduction

Despite the existence of the therapeutic drugs and the widespread use of the subunit recombinant vaccines for prophylaxis, hepatitis B virus (HBV) has been a serious threat to human health until now, particularly in developing countries, with an estimated 370 million chronic carriers (1, 2). Persistent chronic HBV infection may result in cirrhosis and hepatocellular carcinoma, with high mortality rates (1). Hepatitis B patients whose infection has been eradicated develop an effective long-lasting polyclonal, multispecific immune response. This response is not seen in patients with persistent HBV infection, and these patients also show the diminished CD4+ T, responses to hepatitis B virus surface antigen (HBsAg) and weak or undetectable CD8+ CTL responses to multiple hepatitis B epitopes (3, 4). Therefore, the principal goal for the people chronically infected with HBV should be to stimulate a successful immune response, which will result in a long-term viral clearance. These observations have led to the concept of the therapeutic vaccination stimulating specific T cell responses (4, 5).

Although the clinical trial results were controversial and preliminary, HBV subunit recombinant protein vaccines were proved to be effective in some chronic HBV infection patients (6–8). In a clinical trial, vaccination elicited PBMC proliferative responses specific for envelope antigen in 7 of 27 chronic HBV carriers, and these responses were mediated by CD4+ T lymphocytes (6). Compared with recombinant vaccine, DNA vaccine is a better technique to stimulate specific cellular immune responses and is effective in the mouse strains that respond poorly to protein subunit vaccines (5, 9), so it may represent an alternative therapeutic approach for chronic HBV infection. In the phase I clinical trial, HBV PreS2/S DNA vaccine primed antigen-specific T cell responses in healthy people (10). Recently, DNA vaccination with PreS2/S was used for chronic HBV infection therapy in the preliminary clinical trial and showed the exciting results that it induced vigorous T cell responses and eliminated the persistent infection (11).

A classical DNA vaccination plasmid encodes a single antigen; however, this may not offer the ideal strategy for CTL activation because it does not contain the sufficient numbers of CTL epitopes. Epitope-based vaccination, compared with the single-antigen immunization, spans the HLA diversity of a target population and primes immune responses against the
multiple epitopes; moreover, it avoids epitope drift in the case of viral infections and even triggers the required type of immune response (12). Therefore, the polytope DNA vaccine technique was developed (13). In this approach, multiple alphabeta CD8+ CTL epitopes derived from several antigens were conjoined into single artificial constructs in a 'string-of-beads' or linear fashion (13–15). Recently, the polytope DNA vaccination has been shown to induce efficient cellular immunity in different models of viruses and tumors (16–18). Moreover, it primed much stronger CTL responses than those based on the single protein antigen (19). Therefore, the polytope DNA vaccine may have an important advantage over the single-antigen-based DNA vaccines for HBV therapy.

Heat shock proteins (hsp) had once been considered as 'chaperokines’ (20) since they have both chaperon and cytokine abilities (21). No matter how its ability to directly activate dendritic cells was probably due to the contaminant of endotoxin (22, 23), it was confirmed that hsp70 molecule was able to mediate antigen-specific CTL responses by a CD4+ T cell-independent pathway (24–26). In the previous study, hsp73 was proved to enhance CTLs to each epitope in multispecific CD8+ T cell responses by delivering them to processing pathways for MHC class I-restricted presentation in H-2d mice (27). Thus, hsp is an attractive innate adjuvant in vaccine formulations to enhance its immunogenicity for CTLs.

In this study, we designed a DNA vaccine based on multiple CTL and Th epitopes for therapeutic immunization and examined its immunogenicity in H-2d and H-2b MHC-restricted mouse models. To improve antigen-presenting and specific CTL responses, hsp70 molecule was fused to polytope antigen as a genetic adjuvant. Finally, we evaluated its potential therapeutic effects in a HBV transgenic mouse model.

Methods

Vector construction

pCI vector (Promega, Madison, WI, USA) was used to construct DNA vaccine plasmids. An artificial polytope antigen comprised continuous one universal Th Pan DR epitope [(PADRE) sequence] and four HBsAg CTL epitopes (S28–38, S96–203, S172–191 and S208–216). Each CTL epitope was followed by AAA nucleotides encoding lysine. IgG κ chain leader sequence was used as signal peptide, and inserted Kozak sequence at the 5’-terminal of signal peptide as the ribosome-binding site, so it generated the polytope vaccine plasmid, pMulE.

Six overlapping oligos, averaging 60–65 nucleotides in length of 15 nucleotides overlaps, were synthesized (Takara, Dalian, China). The final polytope antigen gene was spliced together using splicing by overlap extension and PCR techniques. Dimers were made of synthetic oligonucleotides 1 and 2, 3 and 4 and 5 and 6 (0.4 pg of each) in 40-μl reactions containing standard 1× Pfu PCR buffer, 0.2 mM deoxyribonucleotide triphosphates and 1 U of Pfu DNA polymerase (hot start at 94°C) using the thermal program: 94°C for 10 s, 42°C for 20 s and 72°C for 20 s for five cycles. After five cycles, the PCR program was paused at 72°C and 20-μl aliquots of reactions 2 and 3 were mixed (reaction 1 was left in the PCR machine) and subjected to another five cycles. At cycle 10, the program was paused again and 20 μl reaction 1 was added to 20 μl reaction from the combined reactions 2 and 3, and another five cycles were completed. Two 20-mer oligonucleotides were used to amplify the gel-purified full-length product using the above reaction mixed at an annealing temperature of 50°C for 25 cycles. The full-length gel-purified PCR fragment was cloned into pCI vector to make the polytope vaccine plasmid, pMulE.

HBsAg gene was cloned into pCI vector and generated the pS plasmid. Mycobacterial hsp70 gene was a gift from Qian Huang (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). hsp70 gene was cloned into pCI vector and generated the pH70 plasmid. For enhancing CTL activity, hsp70 gene was fused to the C-terminal of polytope antigen gene, inserted into the pCI vector, and then generated the fusion plasmid, pMulE/hsp. All the constructs were sequenced to confirm the introduction of the desired change.

Cell line

P815 and EL4 cell lines were obtained from the Chinese Tissue Culture Collection (Shanghai, China). After being transfected with 1 μg plasmid encoding eukaryotically expressed HBsAg (pCDNA-S) with Lipolactamite (Invitrogen, Carlsbad, CA, USA), P815 and EL4 cells were maintained under continuous selective pressure. Therefore, the cloned P815/S and EL4/S cell lines that displayed stable expression of HBV S protein were generated and used as target cells in standard (6-h) 51Cr release assays.

Mice

Female BALB/c (H-2d), C57BL/6 (H-2b) and HBV transgenic mice were kept under the pathogen-free condition in the facility of the University of Science and Technology of China Life Science School. BALB/c and C57BL/6 mice were obtained from National Rodent Laboratory Animal Resources (Shanghai, China). HBV transgenic BALB/c mice, expressing HBV full gene (adr serotype) in mice liver, were purchased from Infectious disease Center of no. 458 Hospital (Guangzhou, China). All the mice used for experiments were at the age of 8 weeks.

DNA immunization

Vaccine plasmids were purified from the transformed Escherichia coli strain DH5α by Wizard PureFaction Plasmid DNA Purification System (Promega) and stored at −70°C. Groups of mice (n = 6) were inoculated into each tibialis muscle with 50 μg of 10 μM cardiotixin (Latoxan, Valence, France) and then after 5 days with 100 μg of plasmid DNA in 100 μl 0.9% NaCl saline. After 1 week, all the mice were boosted with the same immunization methods.

CTL assay

The splenocytes from each vaccinated mouse were harvested after lysing red cells with ACK solution (8.3% NH₄C₁₇ mol l⁻¹ Tris, pH 7.4). The spleen cells were suspended in complete DMEM with 10% FCS. For checking CTL to each epitope, the splenocytes were cultured for 6 days in vitro with recombinant murine IL-2 at the final concentration of 10 IU ml⁻¹ and restimulated by the addition of 1 μg ml⁻¹ of the indicated
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Assay for serum alanine aminotransferase activity

Alanine aminotransferase (ALT) was determined by serum transaminase assay kit (Catachem, Bridgeport, CT, USA). After the first injection, HBV transgenic mice were bled on weeks 0, 2, 4, 6, 8, 10 and 12. A total of 40 μl sera samples was incubated with 200 μl L-alanine and L-ketoglutaramic acid for 30 min at 37°C. Twenty minutes after the addition of 200 μl 2,4-dinitro-phenylhydrazine, 2 ml 0.4 N NaOH was added. UV absorptions were measured at 505 nm. ALT activities were calculated from the standard curve.

Statistics analysis

The experimental data were compared and analyzed by SPSS statistics software.

Results

Epitope selection and vector construction

The polytope plasmid, pMulE, was constructed as described in Method and used for DNA vaccination (Fig. 1). The polytope plasmid consisted of one universal Tα epitope, PADRE, and four continuous CTL epitopes of HBsAg (28, 29) (Ld-CTL epitope S28–39, pr-CTL epitope S198–209 and two Kd-CTL epitopes S172–216 and S209–216). PADRE is a synthetic Tα epitope engineered by introducing anchor residues for the different DR motifs of MHC II into a polyanaline backbone (30) and the resulting peptide binds a variety of DR molecules as well as certain mouse class II alleles, including I-Ab, I-Eb and I-Eb. Every epitope was separated from each other with one lysine (Fig. 1). Such a spacer had previously been shown to minimize cleavage bias that may vary at epitope junctions due to the naturally occurring C- and N-terminal sequences of the minimal epitopes themselves (31). The presence of lysine or arginine at the carboxyl-terminus flanking residue was most frequently associated with strong CTL responses (31).

Polytope plasmids induced CTL responses in vivo

Groups of H-2b BALB/c and H-2b C57BL/6 mice (n = 6) were vaccinated by intramuscular (i.m.) injection with the pCI (100 μg), pS (100 μg) or pMulE (100 μg) vaccine plasmid, respectively. The polytope DNA vaccination induced vigorous CTL responses to the syngeneic HBsAg-expressing target cells in H-2b and H-2b mice; moreover, it resulted in a moderate but significant [using a two-way analysis of variance (ANOVA) of all the data, P = 0.011 in BALB/c mice, P = 0.009 in C57BL/6 mice] increase (averaging ≅22% in BALB/c, 24% in C57BL/6) in CTL responses compared with the single HBsAg DNA immunization (Fig. 2). The splenocytes from the mice vaccinated with the control plasmid had no significant specific target cell lysis.

To determine whether the polytope vaccination could induce CTL responses in vivo to the individual epitopes contained within the polytope plasmid, the splenocytes from each mouse were re-stimulated with the indicated peptide or endogenous processed HBsAg and used in the chromium release assays against the target cells sensitized with the same peptide. The pMulE-vaccinated BALB/c and C57BL/6 mice produced effectors specific for each of the two epitopes presented in

T cell proliferation assay

The spleen cells were harvested after lysing red cells with ACK solution and were cultured in triplicate using 96-well round-bottom plates at 5 × 10^6 cell ml^-1 in 200 μl RPMI 1640 medium containing 5% FCS, and were re-stimulated with 10 μg ml^-1 PADRE peptide. After the 72-h incubation, [3H]thymidine ([3H]TdR) was added (1 μCi per well). The cells were incubated for an additional 16 h, and the [3H]TdR incorporation into DNA was measured. The results were corrected for the background activity (A counts per minute).

Cytokines secretion assay

The splenocytes were prepared and cultured with PADRE peptide in triplicate by the same operation in the proliferation assays. The culture supernatants were collected on the third day. The concentrations of IFN-γ and IL-2 were measured by ELISA using the commercial cytokine assay kit (Diaclone, Besoncon, France); the limit of this detection was 5 pg ml^-1.

Serology assay

The mice were bled after the first injection from tails at different time points. The blood samples were centrifuged at 2700 × g for 20 min and the sera were stored at −70°C for antigen assay. HBsAg in the sera were measured using HBsAg ELISA kit (SABC, Shanghai, China). The sera (diluted at 1 : 10) were added into ELISA plates pre-coated with anti-HBsAg mAb. The plates were incubated at 37°C for 2 h. After washing with phosphate-buffered saline with 0.5% Tween-20 (TBPS) three times, 100 μl peroxidase-conjugated secondary antibody was added to wells and incubated at 37°C for 1 h. After washing with TBPS three times, color was generated by adding o-phenylenediamine dihydrochloride substrates, 100 μl 1 M H_2SO_4 was added and the absorbance at 492 nm was measured on an ELISA reader.

Real-time quantitative PCR assay

The HBV transgenic mice were bled after the first injection and 50 μl sera samples was prepared. HBV DNA was checked by HBV DNA PCR-FLUOTEC kit (SABC). The fluorescent signals were examined by PRISM 7000 Quantitative PCR (ABI, Foster city, CA, USA). HBV DNA was calculated by PRISM 7000 SDS software.

synthetic peptide (Sangon, Shanghai, China) or the irradiated syngeneic HBsAg-expressing transfectants (10^6 irradiated stimulator cell for 3 × 10^7 splenocytes). These effectors were used in standard (6-h) ^51Cr release assays against the peptide-sensitized target cells (P815 for BALB/c and EL4 for C57BL/6). Target cells were sensitized for 1 h with 10 μg ml^-1 peptide at 37°C followed by two washes. For checking CTL to HBsAg, the splenocytes were re-stimulated by recombinant IL-2 at the final concentration of 10 IU ml^-1 and the irradiated syngeneic HBsAg-expressing transfectants. After 5 days, CTLs were harvested and washed. Specific cytolytic reactivity was determined by standard (6-h) ^51Cr release assays against syngeneic HBsAg-expressing target cell, P815/S or EL4/S. In these two assays, serial dilutions of the effector cells were cultured with 2 × 10^3 ^51Cr-labeled targets in 200-μl round-bottom wells in triplicate.
these two mouse strains (Fig. 3). These results demonstrated that polytope DNA vaccine induced independent MHC-restricted CTL responses to the multiple individual epitopes and viral antigen. Surprisingly, when re-stimulated with the irradiated syngeneic HBsAg-expressing P815/S cells, the pMulE vaccination elicited much weaker CTL responses to Dd-CTL epitope S198–209 than those to Ld-CTL epitope S28–39 in BALB/c mice (Fig. 3B and D).

Polytope plasmid induced Th proliferation
The induction of Th is a crucial component of both humoral and cellular immune response. Th secrete cytokines, such as IL-2, that play a fundamental role in the induction and the differentiation of B cell precursors into antibody-forming cells. The secretion of cytokines by Th is also important in the differentiation and the maturation of CTLs. In our vaccine design, a universal helper CD4 T cell epitope, PADRE, was incorporated into the polytope plasmid as CD4 Th activator. The immunogenicity of PADRE was tested by the Tcell proliferation assay. When stimulated with the artificially synthesized PADRE peptide, the splenocytes from the vaccinated mice showed vigorous proliferation (Fig. 4A); moreover, IL-2 and IFN-γ were secreted by these splenocytes in the parallel cytokine assays (Fig. 4B). All these results indicated that CD4 T cell responses were elicited by the polytope vaccine.

CTL responses primed by polytope plasmid were mediated by hsp70
To improve the CTL responses, hsp70 molecule was used as a genetic adjuvant. The hsp70 fused polytope plasmid and the hsp-expressing plasmid were constructed and named as pMulE/hsp and pH70, respectively (Fig. 1C). Groups of BALB/c
Fig. 3. CTL responses to each epitope in BALB/c and C57BL/6 mice. Groups of BALB/c H-2d and C57BL/6 H-2b mice (n = 6) were, respectively, vaccinated by i.m. injection with 100 μg pMulE plasmid (closed circle) and 100 μg pMulE/hsp plasmid (opened circle). The splenocytes from BALB/c (A–D) and C57BL/6 (E–H) mice were re-stimulated in vitro with the indicated CTL epitope peptides or the irradiated syngeneic HBsAg-expressing cells, respectively, and then were used in standard 51Cr release assays against the relevant peptide-sensitized P815 target cells (BALB/c, H-2d effectors) or EL target cells (C57BL/6, H-2b effectors). (A and B) against S28–39-sensitized P815 cells, (C and D) against S198–209-sensitized P815 cells, (E and F) against S172–191-sensitized EL4 cells and (G and H) against S208–216-sensitized EL4 cells. Specific lysis is expressed as percent lysis obtained from peptide-sensitized target cells (+pep) minus the percent lysis obtained using the same effector and target cells in the absence of peptide (−pep) ± SD. The percent lysis values obtained from the latter never exceeded 5%.
and C57BL/6 mice \( (n=6) \) were vaccinated by i.m. injection with pMulE/hsp plasmid (100 \( \mu \)g) and i.m. co-injection with pMulE plus pH70 (100 \( \mu \)g plus 100 \( \mu \)g), respectively. In both BALB/c and C57BL/6 mice, the pMulE/hsp vaccination elicited stronger CTL responses to HBsAg than the pMulE vaccination (Fig. 2). It had an average 40\% increase \( (P=0.005) \) in BALB/c mice and an average 34\% increase \( (P=0.004) \) in C57BL/6 mice (using a two-way ANOVA of all the data) in CTL responses to HBsAg. Moreover, the CTL responses to each epitope were also significantly enhanced (using a two-way ANOVA of all the data, \( P<0.05 \)) when immunized with the hsp-fused polytope plasmid in H-2\(^d\) and H-2\(^b\) mice (Fig. 3). However, when co-injecting the pMulE plasmid with the pH70 plasmid, neither BALB/c nor C57BL/6 mice showed the distinct increased CTL responses (Fig. 2).

Furthermore, the splenocytes from the mice immunized with the pMulE/hsp secreted much more IFN-\( \gamma \) (Student’s \( t \)-test, \( P<0.05 \), \( n=6 \)) than those from the mice immunized with the pMulE in the cytokine secretion assay (Fig. 4B), but these splenocytes only showed little changes in T cell proliferation assay (Fig. 4A) and the secretion of IL-2 had no significant change. These results indicated that the enhanced CTL responses were not due to PADRE-specific CD4 \( T_h \) responses.

Polytope vaccination resulted in the clearance of HBsAg and the down-regulation of HBV DNA replication in a HBV transgenic mouse model

It is known that the clearance and the control of HBV infection are related closely with the host cellular immune responses. Therefore, the potential therapeutic effect of the polytope vaccine was evaluated in the HBV transgenic mice. This mouse model was established by embryonic injection and was tolerant to HBV antigen. Following expression of HBV, viral particles were assembled in liver cells and secreted into blood. Therefore, it was used as a chronic HBV carrier model. Groups of mice \( (n=6) \) were vaccinated by i.m. injection with the pCI (100 \( \mu \)g), pS (100 \( \mu \)g), pMulE (100 \( \mu \)g) or pMulE/hsp plasmid (100 \( \mu \)g), respectively. After the vaccinations, HBsAg significantly dropped in the sera since the first week after immunization in the pS, pMulE and p MulE/hsp groups; moreover, HBsAg were undetectable after 10 weeks (Fig. 5A). Interestingly, the polytope vaccinations, both the pMulE and the pMulE/hsp, resulted in the more rapid down-regulation of HBsAg \( (P<0.05, \) using two-way ANOVA of all the data) than the single HBsAg DNA vaccination. However, anti-HBsAg antibody was not observed by ELISA or western blotting. Furthermore, HBV DNA in the serum was monitored. HBV DNA was distinctly down-regulated and kept at an extremely low level (Fig. 5B). After 6 months, HBsAg was still undetectable and HBV DNA still kept at this low level.

Polytope vaccination induced CTL and \( T_h \) responses in HBV transgenic mice

Since the polytope vaccinations resulted in the significant clearance of HBsAg and down-regulation of HBV DNA, cellular immune responses were checked in the HBV transgenic mice. We found that T cell responses in the transgenic mouse model were similar to those in the BALB/c mouse model. The pMulE vaccination resulted in a moderate but significant (using a two-way ANOVA of all the data, \( P=0.007 \)) increase (averaging \( \approx 30\% \)) in CTL responses to HBsAg-expressing target cells compared with the single HBsAg DNA vaccination in HBV transgenic mice (Fig. 6). Moreover, the
pMulE/hsp vaccination induced stronger CTL responses to viral antigen and each epitope ($P < 0.05$ using two-way ANOVA of all the data) than the pMulE vaccination (Figs 6 and 7). In $T_h$ responses assay, the pMulE and the pMulE/hsp vaccinations caused the significant $T_h$ proliferations and the secretion of IFN-$\gamma$ and IL-2 (Fig. 8). Although the splenocytes from the pMulE/hsp vaccinated mice produced more IFN-$\gamma$ (Student’s $t$-test, $P < 0.05$, $n = 6$) than those from the pMulE-vaccinated mice, no significant change was observed in the secretion of IL-2 and $T_h$ proliferation assays.

**Liver injury assay**

Liver injury was evaluated by checking ALT activities in the sera of the HBV transgenic mice. ALT activity rose after the pMulE/hsp vaccination, and then returned to the normal level and sustained at these levels until the 13th week (Fig. 9). After 6 months, ALT still was kept at this level (data not shown).

**Discussion**

From the history of HBV infection, it is known that the key for therapy of chronic HBV infection is to induce specific and vigorous cellular immune responses, including CD8$^+$ CTL and CD4$^+$ $T_h$ responses. Epitope-based DNA vaccination represents a new therapy strategy for viral infection because of its excellent ability to induce $T_h$ cell responses. DNA vaccine coding multiple continuous CTL epitope can induce specific CTL responses to individual epitope from the different antigens (32), and DNA vaccine coding several $T_h$ epitopes can prime specific $T_h$ responses to each epitope (33). In our research, polytope DNA vaccine containing four CTL epitopes and one universal $T_h$ epitope induced both $T_h$ and CTL responses to each epitope, and it induced much stronger CTL responses to HBsAg than the single-antigen DNA vaccination. Antibody responses against the polytope antigen were not detected in BALB/c or C57BL/6 mice vaccinated by the i.m. injection with the polytope plasmids. Western blotting assay with sera from these mice did not detect a green fluorescent protein (GFP)-fused polytope antigen, while rabbit polyclonal antibody specific for GFP, sc-8334, did recognize the fusion antigen by these assays (data not shown). Surprisingly, we found that the CTL response to S198–209 was much weaker than to S28–29 in BALB/c mice; moreover, it was only significantly primed at the higher effector/target ratio. R. Schirmbeck found that Ld-restricted T cell response (S28–39) to HBsAg efficiently suppressed T cell priming to multiple Dd-, Kd- and Kb-restricted HBsAg epitopes (S198–209, S172–191 and S208–216) in H-2$d^3$b mating mice, but the suppression of Ld-restricted epitope did not hinder the protection function of other epitopes in vivo (34). Therefore, this research provided the reasonable explanation for our results. Nevertheless, this suppression did not affect the CTL responses to Kd- and Kd-restricted epitope in C57BL/6 mice since Ld-restricted epitope was not primed in H-2$d^5$mice.

The previous research showed that the immune responses to a DNA vaccine could be regulated by co-delivery of various cytokine genes (35). These cytokines modulated the immune responses by favoring the development of $T_{h1}$ versus $T_{h2}$. 

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**Fig. 5.** Serology assays in HBV transgenic mice. Groups of H-2$^d$ HBV transgenic mice ($n = 6$) were vaccinated by i.m. injection with pCI (100 µg), pS (100 µg), pMulE (100 µg) or pMulE/hsp plasmid (100 µg), respectively. Mice were bled at different time points after the first injection. Sera were obtained and assayed for the presence of HBsAg (A) by ELISA and HBV DNA (B) by quantitative real-time PCR. The data were presented as mean ± SD for six animals per time point.

**Fig. 6.** CTL responses to HBsAg in HBV transgenic mice. Groups of H-2$^d$ HBV transgenic mice ($n = 6$) were vaccinated by i.m. injection with pCI (100 µg), pS (100 µg), pMulE (100 µg) or pMulE/hsp plasmid (100 µg), respectively. The splenocytes were re-stimulated in vitro with the irradiated syngeneic HBsAg-expressing cells, and then were used in standard 51Cr release assays against P815/S target cells. Specific lysis is expressed as percent lysis obtained from HBsAg-expressing target cells (P815/S) minus the percent lysis obtained using the same effector and parental target cells (P815) ±SD. The percent lysis values obtained from the latter never exceeded 5%.
Therefore, this method provided a strategy to improve the DNA vaccine’s prophylactic and therapeutic efficacy. In our study, hsp70 molecule was used as genetic adjuvant to improve T cell responses since it had both cytokine and chaperone functions. When hsp70 gene was fused to the polytope plasmid, it enhanced the CTL responses in both BALB/c and C57BL/6 mice. However, co-injecting the polytope plasmid with the pH70 plasmid did not result in significant changes in CTL responses. Thus, hsp70 mediated CTL responses by a pathway that was different from cytokines. It was known that hsp70 and hsp60 shared the common receptors that were expressed on human monocyte-derived dendritic cells (36). In H-2d and H-2d mice models, all hsp utilized the CD91R to be internalized by antigen-presenting cells (APCs), and the

**Fig. 7.** CTL responses to each epitope in HBV transgenic mice. Groups of HBV transgenic mice (n = 6) were, respectively, vaccinated by i.m. injection with 100 µg pMulE plasmid (closed circle) or 100 µg pMulE/hsp plasmid (opened circle). The splenocytes were re-stimulated in vitro with the indicated CTL epitope peptides or the irradiated syngeneic HBsAg-expressing cells, respectively, and then were used in standard 51Cr release assays against the relevant peptide-sensitized P815 target cells. (A and B) against S28–39 sensitized P815 cells and (C and D) against S198–209 sensitized P815 cells. Specific lysis is expressed as percent lysis obtained from peptide-sensitized target cells (+pep) minus the percent lysis obtained using the same effector and target cells in the absence of peptide (-pep) ± SD. The percent lysis values obtained from the latter never exceeded 5%.

**Fig. 8.** Th responses in HBV transgenic mice. Groups of HBV transgenic mice (n = 6) were vaccinated by i.m. injection with pCI (100 µg), pMulE (100 µg) or pMulE/hsp plasmid (100 µg), respectively. The splenocytes were re-stimulated in vitro with synthesized PADRE peptide in triplicate, and Th responses were monitored. (A) T cell proliferation assay. After being re-stimulated for 3 days, the splenocytes from transgenic mice were, respectively, used in standard [3H]TdR incorporation assay and the results were expressed as Δ counts per minute corrected for background activity derived from the non-stimulated spleen cells. (B) Cytokine profile of proliferating T cells. The splenocytes were re-stimulated with PADRE peptide as above. The supernatants were collected, and then IL-2 and IFN-γ concentrations (pg/ml^-1) were determined by ELISA. The data were presented as mean ± SD for six animals.
polytope DNA vaccine. As expected, the polytope vaccinations resulted in a long-term clearance of HBsAg. Moreover, hsp70 improved this therapeutic effect as an adjuvant. Nevertheless, HBV DNA was not cleared completely. The trace amount viral gene was still checked out by quantitative PCR, while it was undetectable by northern blotting (data not shown). It suggested that the transgene expression was not extinguished but rather controlled and HBV-specific CTLs suppressed HBV gene expression and replication in the transgenic mice. These results were coincident with the previous study (38). In addition, we found that no obvious liver injury occurred following DNA vaccinations. Furthermore, we evaluated potential therapeutic effects of the polytope DNA plasmids in a HBV transgenic mouse model. As expected, the polytope vaccinations resulted in a long-term clearance of HBsAg. Moreover, hsp70 improved this therapeutic effect as an adjuvant. Nevertheless, HBV DNA was not cleared completely. The trace amount viral gene was still checked out by quantitative PCR, while it was undetectable by northern blotting (data not shown). It suggested that the transgene expression was not extinguished but rather controlled and HBV-specific CTLs suppressed HBV gene expression and replication in the transgenic mice. These results were coincident with the previous study (38). In addition, we found that no obvious liver injury occurred following DNA immunization. Therefore, the viral clearance was mainly due to the non-cytotoxic and lymphokine-based anti-viral mechanism rather than the destruction of infected cell.

HBV has several variations and CTL epitopes not only exist in the surface antigen but also in the core antigen and the polymerase. Although HBV variants with mutations in dominant T cell epitopes might arise during acute hepatitis B infection, they typically remain in low abundance and do not necessarily affect clinical recovery (39). Herein, we chose four CTL epitopes and one T_{h} epitope to design the polytope DNA vaccine, and found that it primed the specific CTL to each epitope and viral antigen in the different MHC-restricted mice models. The polytope vaccine inhibited viral gene replication and cleared the circulating viral antigen in the HBV transgenic mouse model. Furthermore, hsp70 improved the vaccine’s ability to induce specific CTL responses. According to the studies and data presented here, we believe that the polytope DNA vaccine fused with hsp gene may provide an effective therapeutic strategy for chronic HBV carriers.

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Abbreviations
ALT alanine aminotransferase
ANOVA analysis of variance
APC antigen-presenting cell
GFP green fluorescent protein
[^H]Tdr [^H]thymidine
HBV hepatitis B virus
HbsAg hepatitis B virus surface antigen
hsp heat shock protein
i.m. intramuscular
PADRE Pan DR epitope

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