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Construction and immunological evaluation of truncated hepatitis B core particles carrying HBsAg amino acids 119–152 in the major immunodominant region (MIR)

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ABSTRACT

Hepatitis B capsid protein expressed in *Escherichia coli* can reassemble into icosahedral particles, which could strongly enhance the immunogenicity of foreign epitopes, especially those inserted into its major immunodominant region. Herein, we inserted the entire 'α' antigenic determinant amino acids (aa) 119–152 of HBsAg into the truncated HBc (aa 1–144), between Asp^{78} and Pro^{79}. Prokaryotic expression showed that the mosaic HBc was mainly in the form of inclusion bodies. After denaturation with urea, it was dialyzed progressively for protein renaturation. We observed that before and after renaturation, mosaic HBc was antigenic as determined by HBsAg ELISA and a lot of viruslike particles were observed after renaturation. Thus, we further purified the mosaic viruslike particles by $(NH_4)_2SO_4$ precipitation, DEAE chromatography, and Sepharose 4FF chromatography. Negative staining electron microscopy demonstrated the morphology of the viruslike particles. Immunization of Balb/c mice with mosaic particles induced the production of anti-HBs antibody and Th1 cell immune response supported by ELISPOT and CD4/CD8 proportions assay. In conclusion, we constructed mosaic hepatitis core particles displaying the entire 'α' antigenic determinant on the surface and laid a foundation for researching therapeutic hepatits B vaccines.

1. Introduction

Hepatitis B virus (HBV), a widespread and serious human pathogen, has led to more than ~400 million patients having chronic hepatitis B worldwide [1]. Commercial hepatitis B vaccine is immunogenic, safe, and cost-effective in the prevention of HBV infection, but it is ineffective against chronic hepatitis B in which impaired T-cell immune responses to HBV antigens are observed [2]. Hepatitis B surface antigen (HBsAg), present abundantly and persistently in the serum of patients, does not induce anti-HBs antibodies, suggesting that a tolerance to HBsAg exists. However, anti-HBc antibodies are observed in nearly all patients with chronic hepatitis B, indicating that HBc is immunogenic even in the HBsAg-tolerant state. The hapten-carrier effect suggests that if the immunodominant epitope of HBsAg is coupled to HBc, which can stimulate vigorous immune responses including T helper cell (Th cell) responses, the mosaic protein would induce antibody responses to HBsAg and possibly break the tolerance to HBV antigens, for which both hapten-specific B cells and HBc-specific Th cells are required [3].

Moreover, not only can HBc particles induce B-cell, Th-cell, and cytotoxic T-cell (CTL) responses, they have also been demonstrated to permit the inserted epitopes to preserve the intrinsic immunogenicity while protecting experimental animals against challenge with pathogens [4]. Being one of the first icosahedral viruslike particle (VLP) carriers, the HBc particle has been the most flexible and promising model for surface display of foreign epitopes to date [4]. The N-terminal 149 residues of HBc form an assembly domain [5], while the C-terminal 34 residues form a protamine-like domain required for nucleic acid packaging [6]. The assembly domain has an α-helix-rich fold [7], unlike the β-barrel capsid proteins observed in most virus structures [8]. The dimer interface surface spikes are formed by bundles of four α-helices, two from each contributing monomer [9]. HBc is used as a VLP carrier because of its high-level expression and efficient particle formation in virtually all known homologous and heterologous expression systems, including bacteria [10], in which HBc assembles into icosahedral particles that closely resemble those seen in infected liver [11]. In the absence of the protamine-like domain, HBc can

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still assemble into VLPs in *Escherichia coli* [4]. Our previous study also demonstrated that a truncated HBc (aa 1–144) carrying a foreign epitope (aa 21–47 in PreS1) could assemble into icosahedral particles, as supported by electron microscopy [12].

The 'α' antigenic determinant (aa 124–147 in HBsAg) as the major immunodominant epitope is common to all four major serological types of HBV (*adr*, *adw*, *ayr*, and *ayw*) [13] and can induce neutralizing antibodies against HBV [14]. The conformation of the 'α' determinant is essential to the antigenicity of HBsAg [15]. Our previous work demonstrated the immunogenicity of mosaic hepatitis core particles carrying aa 139–148 of HBsAg in the major immunodominant region (MIR) [16], which only keeps one of the three monoclonal antibody epitopes in the 'α' determinant. In the current study, we inserted the entire 'α' determinant including all the three monoclonal antibody epitopes into the MIR of HBc to construct mosaic hepatitis B core particles with the conformational 'α' determinant on the surface.

2. Materials and methods

2.1. Construction of the plasmid expressing mosaic HBc

H5 plasmid containing the HBsAg gene (*adr*) optimized for *E. coli* codon preference was used to amplify the gene encoding aa 119–152 of HBsAg by polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Promega, Madison, WI, USA). The specific primers synthesized by Sangon Biotech (Shanghai, China) were 5′-CGGGATCCGGCCCCTGCAAGA-3′ (forward) and 5′- GCATTACGT AGATGGGGATGCAGG-3′ (reverse). After an initial denaturation at 94 °C for 5 min, all reactions were subjected to 40 cycles at 94 °C for 35 s, 58 °C for 35 s, and 72 °C for 40 s, followed by a final extension at 72 °C for 10 min. After double-enzyme digestion with *BamH*I and *SnaB*I, the gel-extracted 120-bp PCR product was ligated into similarly digested H3 plasmid containing the gene encoding truncated HBc (aa 1–144) between Asp^{78} and Pro^{79} [12]. The ligation reaction mixture was transformed into *E. coli* strain BL21 (DE3). Ampicillin-resistant colonies were isolated and identified by restriction endonuclease analysis of the plasmid, small-scale expression, and sequencing.

2.2. Expression of mosaic HBc

Freshly transformed *E. coli* BL21 (DE3) cells containing the expression plasmid were inoculated into Luria–Bertani (LB) medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) supplemented with 50 μg/ml ampicillin at 37 °C. When the OD_{600} reached 0.8, expression was induced by adding isopropylthio-D-galactoside (IPTG) to a final concentration of 0.8 mM and incubated for an additional 5 h at 28 °C. After harvesting by centrifugation (3000*g*, 10 min, 4 °C), the cell pellet was resuspended in lysate buffer (10 mM Tris–HCl, 0.5% Triton X-100, pH 8.0) and subjected to sonication. The total bacterial proteins, the supernatant, and inclusion bodies were separated by centrifugation (17,400*g*, 10 min, 4 °C) and then subjected to 13.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) to assess the expression and the form of mosaic HBc.

2.3. Purification of mosaic HBc

2.3.1. Renaturation of mosaic HBc

Although the expression level was quite high following the systematic optimization of expression conditions, the mosaic HBc remained mainly in the form of inclusion bodies. The inclusion bodies were resuspended with deionized water and dissolved with denaturation buffer (10 mM Tris–HCl, 8 M urea, pH 8.0). Next, the supernatant was separated by centrifugation (17,400*g*, 10 min, 4 °C) and renatured through three stages of dialysis. The first stage was to gradually remove the urea with renaturation Buffer A (10 mM Tris–HCl, 20 mM L-dithiothreitol (DTT), 300 mM NaCl, pH 8.0). The second stage was to remove the DTT and provide an environment for refolding using Buffer B (10 mM Tris–HCl, 300 mM NaCl, pH 8.0). The last stage was to remove NaCl with Buffer C (10 mM Tris–HCl, pH 8.0).

2.3.2. Purification of mosaic HBc after renaturation

Refolded mosaic HBc was purified by diethylaminoethyl (DEAE) chromatography followed by gel filtration chromatography and finally concentrated by CsCl cushion ultracentrifugation, as described previously [12]. Briefly, refolded mosaic HBc was loaded onto DEAE resin pre-equilibrated with Buffer C, and the nonbinding flow-through fraction was collected. This fraction was concentrated tenfold by incubating overnight with polyethylene glycol 8000 at 4 °C. The supernatant was separated by centrifugation (10,000*g*, 10 min, 4 °C) and subjected to Sepharose 4 Fast Flow chromatography. Fractions (4 ml) were sampled (10 μl) to analyze the distribution of mosaic HBc and assess the homogeneity by 13.5% SDS–PAGE. Finally, the fractions including mosaic HBc with high concentration and purity were pooled and concentrated by 15% CsCl cushion ultracentrifugation (SW40TI rotor, 28,000 rpm, 4 h, 4 °C) in an L-80 ultracentrifuge (Beckman Coulter, Brea, CA, USA). The pelleted viruslike particles were resuspended in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and stored at −4 °C.

2.4. Evaluation of the antigenicity of mosaic HBc by HBsAg ELISA

The HBsAg ELISA Diagnostic Kit was used according to the manufacturer's instructions (WanTai, Beijing, China). Briefly, 100 μl of serially diluted (1:10 to 1:1280) purified mosaic HBc was added to each well and incubated for 60 min at 37 °C. Next, 50 μl of HRP-conjugated antibody solution was added to each well and incubated for 30 min at 37 °C, followed by washing five times with wash buffer. Substrate A (50 μl) and Substrate B (50 μl) were added to each well and then incubated in the dark for 15 min at 37 °C. Finally, 50 μl of Stop solution was added to each well and the OD_{450} and OD_{630} were measured in a spectrophotometer (Thermo Fisher, Vantaa, Finland). The cutoff value was calculated as 2.1 times the mean negative control value (if the value was < 0.05, then the value = 0.05).

2.5. Electron microscopy

Particle preparations following dialysis and purification were negatively stained with uranyl acetate and examined under a Tecnai-G2-12 transmission electron microscope (TEM; FEI, Hillsboro, OR, USA).

2.6. Mice and immunization

Specific-pathogen-free female Balb/c mice aged 6–8 weeks (No. 11400700001060) were purchased from Vital River Laboratories (Beijing, China). All mice were maintained under specific pathogen-free conditions at the Laboratory Animal Center, China CDC, and all studies were approved by the Animal Care and Welfare Committee at the National Institute for Viral Disease Control and Prevention, China Center for Disease Control and Prevention (2013035). To evaluate the mosaic particles, five mice per group were immunized on day 0, 14, and 21 by intramuscular (i.m.) administration of 100 μl of mosaic particles (5 μg/100 μl) or PBS in the control group. Mice were bled by retro-orbital bleeding once every 7 days from day 14 to 56. After standing for 2 h at room

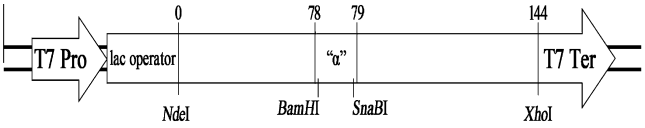

Fig. 1. Schematic diagram of the mosaic HBc gene in pET43.1a vector. T7 pro: T7 promoter; T7 ter: T7 terminator; 'α': aa 119–152 of HBsAg.

temperature (RT), serum was collected by centrifugation (3000*g*, 20 min, RT) and stored at −20 °C. Mice were killed at day 35 and splenocytes were isolated to perform enzyme-linked immunospot (ELISPOT) assays.

2.7. IFN-γ ELISPOT assay

IFN-γ antigen-specific cells were quantified using an ELISPOT kit (BD Biosciences, San Diego, CA, USA). Briefly, splenocytes (5×10^5) were added to multiscreen 96-well filtration plates pre-coated with the anti-mouse IFN-γ capture antibody with 10 μg/ml mosaic particles or 5 μg/ml Con A (positive control) in triplicate wells. The spots were counted using an automated ELISPOT reader. A response was considered to be positive if the number of spot-forming cells (SFCs) per 5×10^5 splenocytes was > 38. SFC values were indicated as the mean ± standard deviation.

2.8. Detection of anti-HBs antibodies by ELISA

The mouse hepatitis B virus surface antibody (HBsAb) ELISA kit was used according to the manufacturer's instructions (Cusabio, Wuhan, China). Briefly, serially diluted serum and HRP-conjugated antibody solution were added to each well and incubated for 30 min at 37 °C. After incubation with Substrate A and Substrate B, the Stop solution was added to quench the reaction and the OD_{450} was measured in a spectrophotometer (Thermo Fisher). The cutoff value was calculated as 2.1 times the mean negative control value (if the value was < 0.05, then the value = 0.05). We regarded the maximum dilution of positive results as antibody titers and used log2 (IgG titers) to analyze the kinetics of the serum anti-HBs antibody.

2.9. Flow cytometry

The relative proportions of $CD4^+$ T and $CD8^+$ T cells in the mouse spleen cells were analyzed by flow cytometry. Briefly, splenocytes (1×10^6/well) derived from mosaic HBc and PBS groups were

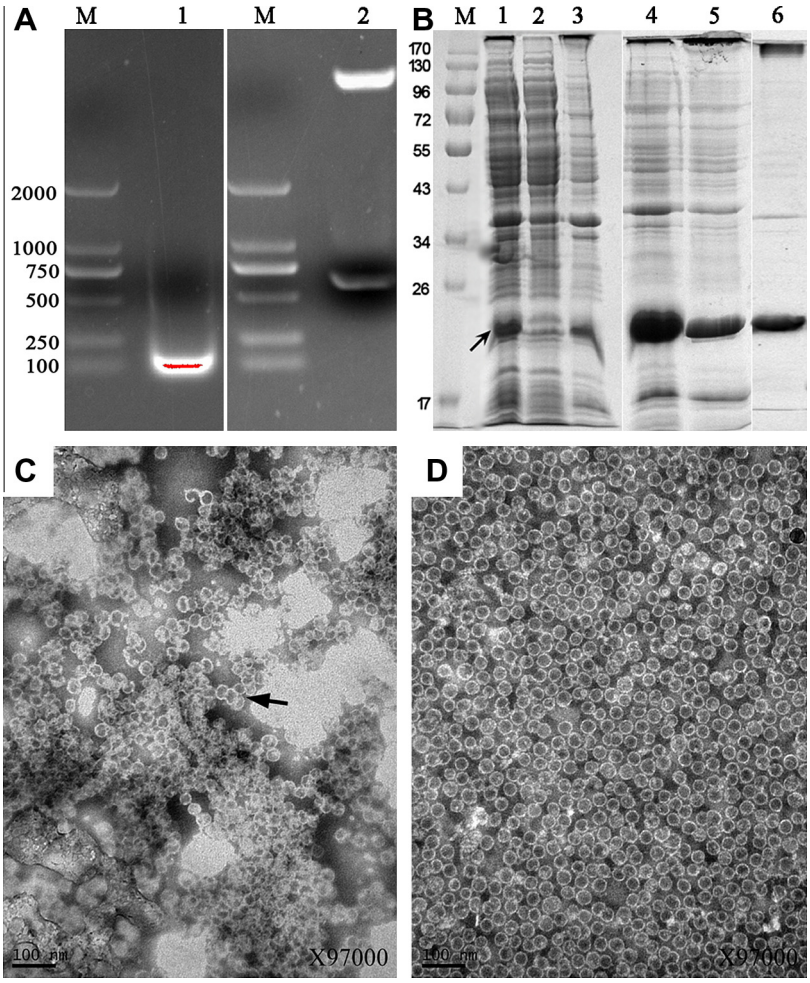

Fig. 2. Agarose gel electrophoresis of the 'α' determinant gene amplified by PCR and restriction endonuclease analysis of the expression plasmid (A) and SDS–PAGE analysis of the expression and purification of mosaic HBc particles (B) and electron microscopy images of mosaic HBc particles (C & D). (A) Lane M, DNA marker DL2000; lane 1, 'α' antigenic determinant gene amplified by PCR; lane 2, expression plasmid digested with *Nde*I/*Xho*I. (B) Lane M, pre-stained protein molecular weight marker; lane 1, total bacterial proteins after sonication of expressing bacteria; lane 2, soluble fraction of the sonicate; lane 3, insoluble fraction of the sonicate; lane 4, inclusion bodies dissolved by urea; lane 5, refolded mosaic HBc particles; lane 6, flow-through fraction from DEAE chromatography. Arrow indicates mosaic HBc. (C) Electron microscope image of refolded mosaic HBc. (D) Electron microscope image of purified mosaic HBc particles.

cocultured for 5 h with 10 μg/ml mosaic particles or PBS alone. Then, 10 μl of FITC-conjugated rat anti-mouse CD8 antibody (eBioscience, San Diego, CA, USA) and PE-conjugated rat anti-mouse CD4 antibody (eBioscience) were added and incubated in a 100-μl volume for 20 min at RT. The splenocytes were washed twice with PBS and resuspended in 500 μl of PBS for flow cytometry (FACScalibur; BD Biosciences).

2.10. Statistical analysis

Statistical analysis was performed using the SNK test. *P*-values of < 0.05 were considered to be statistically significant.

3. Results

3.1. Construction of the plasmid expressing mosaic HBc

We replaced the preS1 neutralizing epitope of the H3 plasmid with the entire 'α' determinant by ligation after double-enzyme digestion with *BamH*I and *SnaB*I (Fig. 1). By PCR, we obtained the gene encoding aa 119–152 of HBsAg, the entire 'α' determinant (115 bp), with *BamH*I and *SnaB*I restriction sites at the termini (Fig. 2A). The expression plasmid was identified by sequencing as well as restriction endonuclease analysis of the plasmid using *Nde*I and *Xho*I (Fig. 2A).

3.2. Expression and purification of mosaic HBc particles

Mosaic HBc was observed mainly in the precipitate following sonication (Fig. 2B), indicating that mosaic HBc was expressed in the form of inclusion bodies. The protein of interest was predominant in the denaturation buffer, although approximately 7/15 were lost in the refolding process (Fig. 2B). Following denaturation with urea and refolding through serial dialysis procedures, the mosaic HBc displayed antigenicity of HBsAg, supported by the HBsAg ELISA assay results (Fig. 3A). With serial dilutions, the slope coefficient of the fitted curve was less than that of common protein, also consistent with a dose–effect relationship. Moreover, large numbers of viruslike particles were observed in the refolded mosaic HBc (Fig. 2C), which was subjected to DEAE chromatography and eluted mainly in the flow-through fraction, indicating that it did not bind to the resin (Fig. 2B). Following Sepharose 4FF chromatography, the mosaic HBc particles were mainly in the major peak after *E. coli* contaminants had eluted in the first peak. The fact that mosaic HBc under nonreducing conditions did not enter the gel suggested its polymeric nature, also supported by electron microscopy in which large numbers of hollow viruslike particles were observed (Fig. 2D).

3.3. Cytokine profile by ELISPOT

The signature cytokines produced by the major $CD4^+$ T-cell subsets are IFN-γ for Th1 cells. Once Th1 cells have developed, they secrete IFN-γ, which promotes more Th1 differentiation. Therefore, we performed IFN-γ ELISPOT assays to evaluate the mosaic HBc-specific T cells that tended to the Th1 cell immune response in the spleens of vaccinated mice. Compared with the PBS group, IFN-γ-secreting splenocytes from the mosaic HBc group following stimulation with mosaic HBc were significantly different ($p < 0.05$; Fig. 4), indicating that mosaic HBc induced Th1 cell immune responses while humoral immunity was stimulated simultaneously (Fig. 3B).

3.4. Kinetics of serum anti-HBs antibody titers induced by mosaic HBc

Without any adjuvants, mosaic HBc particles induced HBsAg-specific antibody immune responses in mice. After three immunizations, the level of antibody titers reached a plateau (Fig. 3B) at day 49–56. Booster immunization seemed to be efficacious, comparing the antibody titer of the initial immunization with that of the third. Moreover, the mosaic HBc yielded a mean IgG1 to IgG2a ratio of ~0.76. IgG1 and IgG2a are regarded as indicators of the induction of Th2 and Th1 responses, respectively. Therefore, a

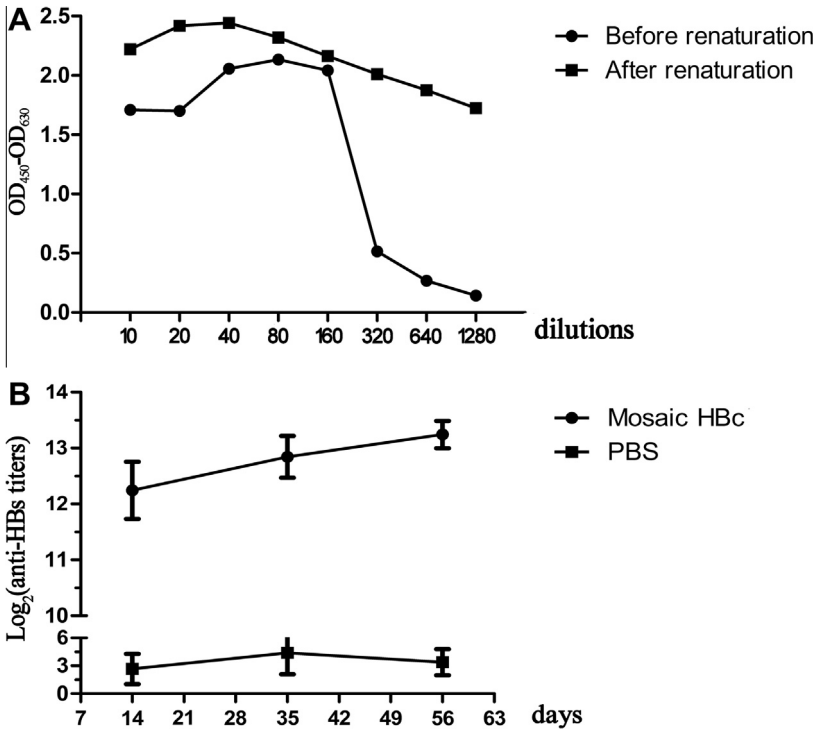

Fig. 3. Antigenicity analysis of mosaic HBc by HBsAg ELISA (A) and kinetics of serum anti-HBs antibody (B).

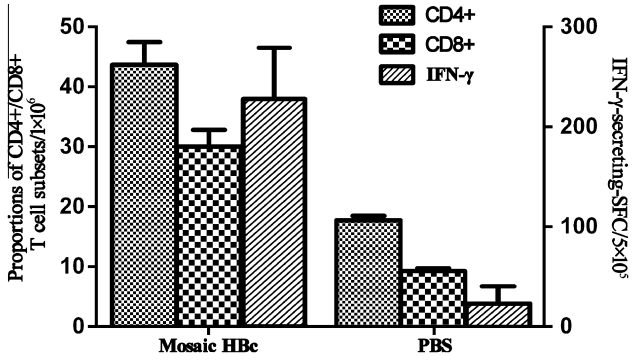

Fig. 4. *In vitro* IFN-γ production by splenocytes of vaccinated mice stimulated with mosaic HBc and proportions of $CD4^+$ and $CD8^+$ T-cell subsets in the splenotype-immunized mosaic HBc and PBS groups.

strong Th1 cell immune response was stimulated by mosaic HBc particles, which was also supported by the ELISPOT assay (Fig. 4).

3.5. Relative proportion of $CD4^+$ and $CD8^+$ T cells

Both $CD8^+$ CTL and $CD4^+$ Th cells are essential for stimulating the immune response of patients with chronic hepatitis B. Flow cytometry analysis indicated that the proportions of $CD4^+$ and $CD8^+$ T-cell subsets were significantly higher in mice immunized with mosaic HBc than those immunized with PBS ($p < 0.05$), suggesting that the conjugated vaccine could activate both $CD4^+$ and $CD8^+$ T-cell subsets. Furthermore, the $CD4^+/CD8^+$ T-cell ratio in mice immunized with the conjugated vaccine was 1.45, compared with 1.91 in the PBS group, indicating that the mosaic HBc induced greater activation of $CD8^+$ T cells than $CD4^+$ T cells (Fig. 4).

4. Discussion

In this study, mosaic HBc particles induced a vigorous immune response in mice, including humoral and cellular immunity. The vaccinated mice produced a high titer of anti-HBs antibody (Fig. 3B) and IFN-γ-secreting T lymphocyte proliferation (Fig. 4).

The appropriate spatial arrangement of identical epitopes presented on the surface of mosaic HBc particles enhanced the immunogenicity of the entire 'α' determinant. Disulfide bonds between certain cysteine residues in the 'α' determinant of HBsAg are important for high-affinity recognition by anti-HBs antibodies [17]. Therefore, the 'α' determinant was employed in its entirety including all of the cysteine residues present in the sequence to allow for a conformation as close to that of the native protein as possible. Although denaturation and renaturation procedures were necessary, we retained three major antigenic epitopes recognized by monoclonal antibodies, including the aa 141–145 epitope. Three intrinsic cysteines exist in the HBc carrier, in which Cys^{48} and Cys^{61} seem to be important to forming the HBc dimer. The entire 'α' determinant possesses seven cysteines, in which two disulfide bonds are necessary to constitute the conformational epitopes of the 'α' determinant between Cys^{124}, Cys^{136} and Cys^{138}, Cys^{147}. Misfolding occurred due to the formation of erroneous disulfide bonds, but most mosaic HBc particles presented the correct conformation of the entire 'α' antigenic determinant, supported by the antigenicity of mosaic HBc particles (Fig. 3A), and assembled into viruslike particles (Fig. 2C) after renaturation. First, we employed DTT to break all of the disulfide bonds and gradually reduced the concentration of DTT to ensure the formation of disulfide bonds according to the principle of minimum free energy because the two cysteines forming a disulfide bond have a priority to reduce the intrinsic energy through disulfide bond formation.

T-cell epitopes of HBc also contributed to enhancing the immunogenicity of the 'α' determinant and activation of cellular immunity. HBc possesses many Th cell epitopes and HLA-restricted CTL epitopes, in which especially, the aa 50–69 epitope can bind with different HLA-II molecules and be recognized by 70–80% of patients with chronic hepatitis B or nonresponders [4]. Helper T cells activated by Th and CTL epitopes of HBc could provide second signals for the development of plasmocytes and $CD8^+$ T cell-specific B cells, and promote antibody production and differentiation into functional CTLs. Without any adjuvants, HBc particles can induce Th1 cell responses [18] and enhance the immunogenicity of HBc through T–B cell interactions [19]. We employed these features to construct mosaic HBc particles that simultaneously induced the production of anti-HBs antibody and differentiation of Th1 cell responses.

In addition, mosaic HBc particles could bind anti-HBc antibody *in vitro*, supported by the Western blotting assay results [12], suggesting that the same event will occur *in vivo* with antigen–antibody complex (IC) formation, which was originally used to enhance the immunogenicity of specific antigens to raise high titers of antibodies in animals [20]. The IC can modulate cellular and humoral immune response by opsonizing the phagocytosis of mosaic HBc and by activating the complement system by exposing the Fc region of the antibody, which is therefore more immunogenic than the antigen alone. Phagocytosis of mosaic HBc promotes the presentation and processing of antigens including inserted foreign epitopes, which is important for the activation of effector immune cells. Wen et al., demonstrated that the tolerogen (HBsAg) would be forced to be taken up by antigen presenting cells (APCs) with Fc receptors of a complex of HBsAg with anti-HBs in the tolerant host, and that the processing and presentation of HBsAg could be modulated [21].

The evaluation of the immune effect of mosaic HBc carrying the antigenic 'α' determinant in the animal model of chronic hepatitis B needs further study. In normal Balb/c mice, mosaic HBc induced the production of anti-HBs antibody and Th1 cell immune response, supported by HBsAb-ELISA and IFN-γ-ELISPOT assays, respectively. For a therapeutic vaccine, inducing T-cell responses is most important, especially a $CD8^+$ T-cell response, which requires cross-presentation of the exogenous vaccine antigens by professional APCs mediated by antigen uptake into distinct endosomal compartments [22]. The combination of HBsAg and HBcAg induced broad T-cell responses and even displayed an additive effect in inducing anti-HBs seroconversion in HBVtg mice [23]. This additive effect may be related to the ability of HBcAg particles to directly activate B cells to take up the antigen, then process and present it [24]. In this study, we combined the strong T-cell epitopes of HBcAg with strong B-cell epitopes of HBsAg to construct mosaic HBc particles of which each monomer contains the two. Hopefully, the desired responses will appear in patients with chronic hepatitis B immunized with mosaic HBc particles.

References

[1] W.M. Lee, Hepatitis B virus infection, N. Engl. J. Med. 337 (1997) 1733–1745.
[2] K. Kakimi, M. Isogawa, J. Chung, A. Sette, F.V. Chisari, Immunogenicity and tolerogenicity of hepatitis B virus structural and nonstructural proteins: implications for immunotherapy of persistent viral infections, J. Virol. 76 (2002) 8609–8620.
[3] N.E. Harwood, F.D. Batista, New insights into the early molecular events underlying B cell activation, Immunity 28 (2008) 609–619.
[4] P. Pumpens, E. Grens, HBV core particles as a carrier for B cell/T cell epitopes, Intervirology 44 (2–3) (2001) 98–114.
[5] F. Birnbaum, M. Nassal, Hepatitis B virus nucleocapsid assembly: primary structure requirements in the core protein, J. Virol. 64 (7) (1990) 3319–3330.
[6] D.R. Milich, A. McLachlan, S. Stahl, P. Wingfield, G.B. Thornton, J.L. Hughes, J.E. Jones, Comparative immunogenicity of hepatitis B virus core and E antigens, J. Immunol. 141 (10) (1988) 3617–3724.

[7] P.T. Wingfield, S.J. Stahl, R.W. Williams, A.C. Steven, Hepatitis core antigen produced in *Escherichia coli*: subunit composition, conformational analysis, and in vitro capsid assembly, Biochemistry 34 (15) (1995) 4919–4932.
[8] M.G. Rossmann, J.E. Johnson, Icosahedral RNA virus structure, Annu. Rev. Biochem. 58 (1989) 533–573.
[9] S.A. Wynne, R.A. Crowther, A.G. Leslie, The crystal structure of the human hepatitis B virus capsid, Mol. Cell 3 (6) (1999) 771–780.
[10] M. Pasek, T. Goto, W. Gilbert, B. Zink, H. Schaller, P. MacKay, G. Leadbetter, K. Murray, Hepatitis B virus genes and their expression in *E. coli*, Nature 282 (5739) (1979) 575–579.
[11] J.M. Kenney, C.H. von Bonsdorff, M. Nassal, S.D. Fuller, Evolutionary conservation in the hepatitis B virus core structure: comparison of human and duck cores, Structure 3 (15) (1995) 1009–1019.
[12] Q.D. Su, M.Z. Guo, Y. Yi, S.Y. Chen, Z.Y. Jia, X.X. Lu, F. Qiu, S.L. Bi, Antigenic analysis of two chimeric hepatitis B core particles presenting the preS1 neutralizing epitopes, Chin. J. Exp. Clin. Virol. 27 (4) (2013) 251–254.
[13] P. Tiollais, C. Pourcel, A. Dejean, The hepatitis B virus, Nature 317 (6037) (1985) 489–495.
[14] J.A. Waters, S.E. Brown, M.W. Steward, C.R. Howard, H.C. Thomas, Analysis of the antigenic epitopes of the Hepatitis B surface antigen involved in the induction of a protective antibody response, Virus Res. 22 (1991) 1–12.
[15] S. Mishiro, M. Imai, K. Takahashi, A. Machida, T. Gotanda, Y. Miyakawa, M. Mayumi, A 49,000-dalton polypeptide bearing all antigenic determinants and full immunogenicity of 22-nm hepatitis B surface antigen particles, J. Immunol. 124 (4) (1980) 1589–1593.
[16] S.Y. Chen, M.Z. Guo, F. Qiu, Y.L. Fei, Y. Yi, Y. Guo, Z.Y. Jia, T. Yu, S.L. Bi, Construction and characterization of hepatitis B surface antigen "α" epitope virus-like particles, Chin. J. Exp. Clin. Virol. 24 (1) (2010) 30–32.
[17] S.E. Brown, C.R. Howard, A.J. Zuckerman, M.W. Steward, Determination of the affinity of antibodies to hepatitis B surface antigen in human sera, J. Immunol. Methods 72 (1) (1984) 41–48.
[18] T. Fehr, D. Skrastina, P. Pumpens, R.M. Zinkernagel, T cell-independent type I antibody response against B cell epitopes expressed repetitively on recombinant virus particles, PNAS 95 (16) (1998) 9477–9481.
[19] U. Lazdina, T. Cao, J. Steinbergs, M. Alheim, P. Pumpens, D.L. Peterson, D.R. Milich, G. Leroux-Roels, M. Sällberg, Molecular basis for the interaction of the hepatitis B virus core antigen with the surface immunoglobulin receptor on naïve B cells, J. Virol. 75 (14) (2001) 6367–6374.
[20] G. Terres, W. Wolins, Enhanced immunological sensitization of mice by the simultaneous injection of antigen and specific antiserum. I. Effect of varying the amount of antigen used relative to the antiserum, J. Immunol. 86 (1961) 361–368.
[21] Y.M. Wen, Z.H. Yuan, X. Yao, Experimental and clinical studies on hepatitis B surface antigen-antibody complex therapeutic vaccine, in: D.V. Denyer (Ed.), Progress in Hepatitis B Research, Nova Science Publishers, Inc., New York, 2007, pp. 203–216.
[22] S. Burgdorf, A. Kautz, V. Bohnert, P.A. Knolle, C. Kurts, Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation, Science 316 (5824) (2007) 612–616.
[23] W. Kastenmuller, G. Gasteiger, J.H. Gronau, R. Baier, R. Ljapoci, D.H. Busch, I. Drexler, Cross-competition of CD8+ T cells shapes the immunodominance hierarchy during boost vaccination, J. Exp. Med. 204 (9) (2007) 2187–2198.
[24] D.R. Milich, M. Chen, F. Schodel, D.L. Peterson, J.E. Jones, J.L. Hughes, Role of B cells in antigen presentation of the hepatitis B core, PNAS 94 (26) (1997) 14648–14653.