Immune responses to recombinant *Mycobacterium smegmatis* expressing fused core protein and preS1 peptide of hepatitis B virus in mice

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**Abstract**

Attenuated strains of bacteria have been developed as potential live vectors to express homologous or heterologous antigens of many pathogens for inducing protective immune responses. The non-pathogenic and rapidly growing *Mycobacterium smegmatis* can be transformed effectively by genes for pathogenic antigens, and has been used as a valuable vector for the development of live vaccines. However, little is known on whether *M. smegmatis* could be transformed with the genes for HBV antigens and could express those genes, and whether vaccination with recombinant *M. smegmatis* could induce humoral and cellular immune responses in *vivo*. Both the core protein and preS1 peptide of the hepatitis B virus (HBV) are immunogenic and can induce cellular and humoral immune responses. This made them ideal platform for the development of new vaccines. In the present study, both recombinant *M. smegmatis* and DNA vaccines were generated to express the CS1 antigen, a fusion protein that comprises truncated core protein (amino acids 1–155) and preS1 peptide (amino acids 1–55) of HBV. Following vaccination of BALB/c mice with the live recombinant *M. smegmatis*, the CS1-based DNA vaccine, or controls, antigen-specific humoral and cellular immune responses were characterized. Vaccination with live recombinant *M. smegmatis* induced a stronger cellular immune response and a longer period of humoral immune response than with the DNA vaccination. These results indicate that the recombinant *M. smegmatis* can express efficiently immunogenic CS1 antigen of HBV in *vivo*, and may be used for the prevention of HBV infection.

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**1. Introduction**

Infection with hepatitis B virus (HBV) is associated with liver cirrhosis, hepatocellular carcinoma and other complications. Hepatitis B virus is prevalent in Africa, Southern Europe, Latin America, and especially in Eastern Asia including China. Some two billion people worldwide show serological evidence of infection with this virus, and approximately 5–10% of adults become chronic carriers of HBV. Importantly, 80–90% of children infected with HBV early in life may progress to chronic liver diseases (Kao and Chen, 2002; Yuen and Lai, 2001).

Current treatment for chronic hepatitis is far from satisfactory. Lamivudine or nucleoside analogues are able to reduce HBV DNA to undetectable levels. However, the suspension of treatment usually leads to a rapid relapse of the infection. In addition, a long-term administration of lamivudine often induces drug-resistant viral variants by the mutation of YMDD motif of the HBV polymerase gene (Enomoto et al., 2006; Kao and Chen, 2002; Liaw, 2005; Thio, 2003). Alpha-interferon, although more effective, is more expensive. Prevention of hepatitis caused by HBV is now achieved mainly by immunisation with recombinant hepatitis B surface antigen (HBsAg) produced in yeast or mammalian cells. However, about 5–10% of immunized individuals,
do not develop or develop low levels of antibody to HBsAg (Sjogren, 2005; Woo et al., 2001). Furthermore, people vaccinated with HBsAg can still be infected with the HBV mutants. Moreover, due to the unsatisfactory efficacy of treatment with interferon or lamivudine in patients with chronic infection, it is important to develop new and more effective treatment. Therapeutic specific recombinant protein vaccine, DNA vaccines, and peptide vaccines have been developed (Engler et al., 2001; Heathcote et al., 1999; Mancini-Bourgine and Michel, 2005; Pol et al., 2000). However, their effects are limited. Thus, the development of new vaccines with novel strategies is required.

The conserved core protein of HBV contains determinants for CTL recognition and conformational epitopes for B cell responses, which serves as a potential antigen for the development of new vaccines. Proteins encoded by the preS1 gene, which is responsible for the attachment of the virus, can induce neutralization antibodies in vivo (De Falco et al., 2001). Recent studies have shown that either the core gene or the preS1 gene can be employed for the development of new vaccines against HBV (Beckbebaum et al., 2003; Kakimi et al., 2002; Townsend et al., 1997). However, little is known about the impact of the fusion of both proteins on their immunogenicity and antigenicity in vitro and in vivo.

*M. smegmatis*, a non-pathogenic member of the *Mycobacterium* family, grows rapidly, and can be transformed effectively by many genes in vitro. Currently, it has been employed to develop vaccines against various micro-pathogens (Averill et al., 1993; Delogu et al., 2004; Eremeev et al., 1996). Whether *M. smegmatis* could be transformed effectively with the genes for truncated core protein and preS1 peptide and express subsequently those genes, and whether the vaccination with recombinant *M. smegmatis* could induce humoral and cellular immune responses remains to be determined.

In this study, eukaryotic and prokaryotic expression vectors were constructed and recombinant *M. smegmatis* carried the fused genes for truncated core protein and preS1 peptide of HBV was generated. After inoculation with recombinant *M. smegmatis* or recombinant DNA plasmids, which expressed the fused proteins in vitro, antigen-specific cellular and humoral immune responses were characterized. The findings of this study provide new insights into the possibility of *M. smegmatis* as a vehicle for expression of HBV antigens and to aid in the development of new vaccines against HBV.

2. Materials and methods

2.1. Amplification of the genes for truncated core and preS1

The truncated core gene of HBV encoding for amino acids 1-155 and the gene for amino acids 1-55 of preS1 were amplified by PCR from plasmid pCP10 (Mount Sinai Medical Center, New York, NY, USA), which carries the genome of HBV (adw subtype) using two pairs of specific primers, respectively. The primers for the amplification of truncated core gene were 5′-GCGGTGATGGGGCGAGGCATCCTTCCGAC-3′ (forward primer) and 5′-GCCCCTGAGGCACGCTGATCC-3′ (reverse primer). The primers for the preS1 gene were 5′-GCGCTGCAGATGGGGCGAGGCATCCTTCCGAC-3′ (forward primer) and 5′-GCGGCTGATGGGGCGAGGCATCCTTCCGAC-3′ (reverse primer). After an initial denaturation step at 94 °C for 1 min, all reactions were subjected to 35 cycles of annealing at 56 °C for 50 s, extension at 72 °C for 1 min and denaturation at 94 °C for 50 s.

2.2. Construction of vectors carrying CS1 genes

The purified DNA fragments encoding the truncated core protein were fused with the preS1 region in the same open-reading frame and subcloned into prokaryotic or eukaryotic expression vectors, pET28a (Novagen, Madison, MI, USA), pcDNA3.1(−) (Invitrogen, Carlsbad, CA, USA), or pDE22 (a shuttle secretory plasmid for *M. smegmatis*, our unpublished data), generating plasmids pET28a-CS1, pcDNA3.1(−)-CS1, or pDE22-CS1, respectively. The authenticity of the fused genes was confirmed by DNA sequencing.

2.3. Expression of CS1 fusion protein in Escherichia coli

The pET28a-CS1 were transformed into *E. coli* BL21 cells and cultured in solid Luria–Bertani (LB) medium containing kanamycin (50 mg/L). After propagation, single clones of pET28a-CS1 positive *E. coli* BL21 cells were selected and cultured in LB broth with kanamycin (50 mg/L). The cells were incubated at 37 °C and when their optical density (OD 600) value reached 0.3–0.4, IPTG was added to the broth at the final concentration of 1 mM to induce the expression of the CS1 genes for 3 h. After harvest, the BL21 cell pellet was re-suspended in a lysis buffer containing lysozyme and subjected to sonication. The supernatant and inclusion body pellet were separated by SDS-PAGE analysis. The expressed CS1 protein was purified by Ni2+-NTA column (Qiagen, Düsseldorf, Hilden, Germany) according to the manufacturer’s instruction.

2.4. Western blot analysis of the expressed CS1 protein

The expressed CS1 protein was subjected to SDS-PAGE, and then transferred onto the nitrocellulose membrane (Gibco, Rockville, MD, USA) at 100 V in transfer buffer for 1 h. The bound proteins were subsequently probed with a mouse anti-HBcAg antibody (Santa Cruz, Delaware Avenue, CA, USA) at 1:1000 dilution and subsequently probed with a rabbit anti-mouse IgG-IRDye800 antibody (Odyssey, Li-Cor, Lincoln, NE, USA).

2.5. Analysis of antigenicity of the CS1 fusion protein by enzyme-linked immunosorbent assay

The potential of CS1 protein was evaluated as a diagnostic antigen by ELISA using sera from patients with hepatitis B. Briefly, 96-well ELISA plates were coated with the purified CS1 protein (1 µg well−1) and incubated at 4 °C overnight. After being washed with phosphate buffered saline and blocked with 5% lipid-free milk at room temperature for 1 h, the plates were added with different dilutions of anti-HBcAg or anti-preS1
sena and incubated at 37 °C for 1 h. The bound immunoglobulin (Ig) was characterized using horseradish peroxidase conjugated goat anti-human Ig secondary antibody and tetramethyl benzidine. All the patients signed informed consent with a protocol approved by the Human Protection Committee of Hospital.

2.6. Eukaryotic expression of the CS1 fusion protein and establishment of stable cell lines

P815 cells (CTCC, China Typical Culture Collection Centre, Beijing, China) were incubated in the RPMI1640 medium in 24-well plates. The cells at approximately 90% confluence were transfected with pcDNA3.1(−)-CS1 or control plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h incubation, the transfected cells were harvested and their CS1 expression was characterized by a reverse transcriptase polymerase chain reaction (RT-PCR) and immunofluorescent assay (IFA). In brief, total RNA was extracted from transfected cells with 1 mL TRIZOL reagent (Gibco, Rockville, MD, USA) and reverse transcribed into cDNA using random primers (Promega, Madison, WI, USA). The cDNA was amplified by PCR using specific oligonucleotide primers as described in Section 2.1.

The transfected cells were fixed by paraformaldehyde, and blocked with BSA, and then incubated with a mouse anti-HBc antibody (HBcAb) (Santa Cruz, Delaware Avenue, CA, USA) at 4 °C overnight. After being washed, the cells were incubated with the secondary antibodies conjugated with FITC followed by examination by fluorescence microscopy (Olympus, Kyoto, Japan).

The remaining transfected cells were cultured in the presence of 350 μg/mL neomycin (G418, Gibco, Rockville, MD, USA). Single cell clones were isolated and their CS1 expression was characterized by RT-PCR and IFA.

2.7. Bacterial strains and transformation by electroporation

M. smegmatis (M. smegmatis mc² 155, Institute of Tuberculosis in Shanxi, Xi’an, China) was cultured in Luria–Bertani (LB) medium and transformed with a shuttle vector, pED22-CS1, or a control vector as previously described (Averill et al., 1993; Delogu et al., 2004; Eremeev et al., 1996). Transformed strains were plated in solid LB medium containing hygromycin (Roche, New York, NY, USA). Typically transformed bacterium clones from the plates were transferred into 50 mL of LB medium with 5% Tween-80 and hygromycin (50 mg/L), and cultured for 5 days. Recombinant M. smegmatis was harvested and identified by PCR using the primers as described in Section 2.1.

2.8. SDS-PAGE and Western blot analysis of the expressed protein in M. smegmatis

The CS1+ recombinant bacterial strains were incubated in LB medium at 37 °C. Before harvest, they were heat-induced at 42 °C for 30 min for the expression of the fused genes. The supernatant and sediments were collected separately. The supernatant was dialyzed against 8 mol/L urea and PBS, and then concentrated by PEG 6000 (Sigma, Saint Louis, MO, USA). The supernatant products and sediments were analyzed by SDS-PAGE and Western blotting as described in Section 2.4.

2.9. Immunization of mice with pcDNA3.1(−)-CS1 and recombinant M. smegmatis

Male BALB/c mice at 4–6 weeks of age were from the Medical Experimental Animal Center of Fourth Military Medical University (Xi’an, China) and maintained under specific pathogen-free conditions. Groups of 10 mice were immunized intradermally with 10⁶ CFU recombinant M. smegmatis or control M. smegmatis in 0.1 mL of PBS for the evaluation of immune responses to immunized antigens. Additional groups of mice were injected intramuscularly with 100 μg of pcDNA3.1(−)-CS1 or control pcDNA3.1(−). The mice were boosted with the same injectant 1 month later. Blood was collected from the caudal vein every 15 days up to 75 days post immunization for analysis of humoral responses. All the experimental protocols were approved by the Research Animal Administration of the Fourth Military Medical University.

2.10. Analysis of the humoral response

Antibodies in sera from immunized mice were tested by antigen-specific ELISA as described in Section 2.5. Briefly, a micro-well plate was coated with the CS1 protein purified from recombinant E. coli. Antibody titers were determined by the serial end-point dilution method.

2.11. Analysis of T cell response

Antigen-specific T cell proliferation was evaluated by 3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma, Saint Louis, MO, USA) assay. Splenocytes (1 × 10⁷ well⁻¹) isolated from the vaccinated mice were stimulated with the purified CS1 fusion protein (2 μg/mL) for 54 h, and exposed to MTT (5 mg/mL) for 4 h. Splenocytes incubated with medium alone were used as a negative control. After the medium was removed, 150 μL methyl sulfoxide (Sigma, Saint Louis, MO, USA) was added into each well of the plate, and the plate was incubated at 37 °C for 10 min, followed by the measurement of the optical value using the microplate reader. Antigen-specific proliferation index (PI) was calculated by the relative value of experimental groups over that of the negative controls.

Splenocytes isolated from experimental or control mice were used as the effector, and P815 cell clone expressing CS1 was used as the antigen. Antigen-specific cytotoxic activity was determined by lactate dehydrogenase (LDH) assay according to the protocol of the manufacturer (Promega, Madison, WI, USA).

2.12. Statistical analysis

The significance of the difference in various parameters between different groups was statistically analyzed by SPSS 10.0.
3. Results

3.1. Prokaryotic expression and purification of the CS1 fusion protein

The prokaryotic vector, pET28a-CS1, was constructed by inserting the genes for truncated core and preS1 behind T7 promoter of pET28a, allowing IPTG-induced CS1 expression in prokaryotic cells. Subsequently, whether the constructed pET28a-CS1 could express effectively the fused CS1 protein in prokaryotic cells was tested. pET28a-CS1 was transformed into E. coli BL21 and single clones of bacteria were cultured before they were treated with IPTG for the induction of CS1 expression. After harvesting, different clones of bacteria containing pET28a-CS1 or control vector alone as well as extracted inclusion body or remaining supernatants were characterized for the CS1 expression by SDS-PAGE. First, without IPTG induction, the profile of protein in E. coli BL21 clone that had been transformed with pET28a-CS1 was similar to that of control E. coli BL21 clone (Fig. 1a). Following IPTG induction, a strong protein band displayed the expected molecular weight (about 23 kDa), indicating the CS1 expression. Furthermore, while supernatants of sonicated bacteria showed little CS1 expression, the extracted inclusion body presented a strong protein band with the expected size. Western blot analysis revealed that the expressed CS1 antigen could bind effectively to the anti-HBcAg mAb, similar to that of the purified CS1 protein (Fig. 1b). These data indicate that the purified CS1 protein can bind effectively not only to mouse HBcAg mAb but also to antibodies developed naturally in humans.

3.2. Antigenicity of the CS1 protein

To address further whether the purified CS1 protein could be used for the detection of anti-HBc antibodies in the clinic, ELISA was employed for testing of 24 sera from HBcAb positive patients and 24 sera from anti-preS1 positive patients, using the purified CS1 as the coated antigen. When the purified CS1 protein was used as the antigen, 23 out of 24 HBcAb positive sera (mean OD value was 1.30 ± 0.09, n = 24) and 22 out of 24 anti-preS1 positive sera (mean OD value was 1.17 ± 0.11, n = 24) displayed a strong signal of antibody binding. In addition, with the purified CS1 protein, positive sera up to 640 folds of dilution were detected (mean antibody titer value was 1:348 ± 16, n = 20). These data indicate that the purified CS1 protein can bind effectively not only to mouse HBcAg mAb but also to antibodies developed naturally in humans.

3.3. Eukaryotic expression of the CS1 fused genes

To express the CS1 genes in eukaryotic cells, the CS1 DNA fragment was subcloned behind the CMV promoter in pcDNA3.1(−) vector to generate pcDNA3.1(−)-CS1 plasmid. After demonstrating its sequence, these plasmids were transfected into P815 cells, respectively, and the CS1 expression in P815 cells was characterized by RT-PCR and IFA. Cells transfected with pcDNA3.1(−)-CS1, but not with the empty vector pcDNA3.1(−), displayed transcripts (data not shown). The expressed CS1 protein was recognized by HBcAg-specific mAb, and was primarily located in the cytoplasm of the cells (Fig. 2). These data indicate that the constructed pcDNA3.1(−)-CS1 expresses effectively the CS1 protein in eukaryotic cells and can be used for DNA vaccination in vivo.

3.4. Expression of the CS1 fusion protein in recombinant M. smegmatis

M. smegmatis has been employed recently as the vehicle to develop live vaccines for the induction of immune responses against many other pathogens. However, whether it could be used for the generation of recombinant vaccine against HBV has never been tested. To address this issue, the CS1 fused genes were subcloned into the pDE22, a shuttle vector, generating the pDE22-CS1 plasmid. Following sequence analysis, the pDE22-CS1 was transformed into M. smegmatis by electroporation, leading to the expression and secretion of the CS1 protein following the heat-induction of recombinant M. smegmatis-pDE22-CS1. Control pDE22 was transformed into M. smegmatis to form the control recombinant M. smegmatis-pDE22. Four positive clones of recombinant M. smegmatis-pDE22-CS1 as well as controls, which were hygromycin-resistant with typical colony phenotype, were isolated. The preS1 gene, truncated core gene, and CS1 fusion genes were amplified only from recombinant M. smegmatis-pDE22-CS1 by PCR using the specific primer (data not shown). The analysis of the CS1 expression revealed that the fused CS1 protein from recombinant M. smegmatis-pDE22-CS1 was displayed...
Fig. 2. Immunofluorescence assay of stable cell lines expressing CS1. P815 cells were transfected with pcDNA3.1(−)-CS1 or control vector, and treated with G418 to generate stable cell line. Their CS1 expression was characterized by immunofluorescent assay using FITC-anti-HBcAg and Evan’s dyestuff. (a) P815 cells transfected with pcDNA3.1(−)-CS1 (200x). (b) P815 cells transfected with empty pcDNA3.1(−) vectors (400x). Data are a representative of five independent experiments.

Fig. 3. Analysis of CS1 expression by recombinant *M. smegmatis*. (a) SDS-PAGE analysis of CS1 fusion protein. MW, protein molecule weight markers; lanes 1 and 2, the lysates of recombinant *M. smegmatis* with heat-induction; lane 3, lysates of recombinant *M. smegmatis* without heat-induction; lane 4, control *M. smegmatis* with heat-induction; and lane 5, the supernatant of recombinant *M. smegmatis* culture with heat-induction. The expressed CS1 protein with molecular weight about 23kDa was pointed by an arrow. (b) Western blot analysis of CS1 protein from recombinant *M. smegmatis*. Lane 1: the sediments of recombinant *M. smegmatis* with heat-induction; lane 2, the supernatant of recombinant *M. smegmatis* with heat-induction; lane 3, the lysates of control *M. smegmatis* with heat-induction; and lane 4, the lysate of recombinant *M. smegmatis* without heat-induction. Data represent five independent experiments.

3.5. Humoral response to the CS1

Groups of BALB/c mice were injected intradermally with recombinant *M. smegmatis*-pDE22-CS1, control recombinant *M. smegmatis*-pDE22, or a wild type of *M. smegmatis*. Additional groups of mice were vaccinated intramuscularly with pcDNA3.1(−)-CS1 or control pcDNA3.1(−) or injected with saline. Following immunization, humoral responses to the CS1 were characterized longitudinally by ELISA. Notably, while all the mice vaccinated with recombinant *M. smegmatis* survived throughout the experimental period, they developed skin lesions, with typical tubercula, in the injection sites. The distinct skin lesions appeared 3 days after immunization, which maintained throughout the experimental period, indicating the successful

<table>
<thead>
<tr>
<th>Table 1</th>
<th>CS1-specific antibody titers in mouse sera</th>
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<tr>
<td>Groups</td>
<td>15 Days</td>
</tr>
<tr>
<td>Saline</td>
<td>10.2 ± 1.3</td>
</tr>
<tr>
<td>pcDNA3.1(−)</td>
<td>40.4 ± 2.5</td>
</tr>
<tr>
<td>pcDNA3.1(−)-CS1</td>
<td>5228.1 ± 21.1</td>
</tr>
<tr>
<td>Smegmatis</td>
<td>70.2 ± 5.4</td>
</tr>
<tr>
<td>Smegmatis + pDE22</td>
<td>75.0 ± 6.8</td>
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<tr>
<td>Smegmatis + pDE22-CS1</td>
<td>2469.5 ± 34.4</td>
</tr>
</tbody>
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*p < 0.05. Antibody titer of pcDNA3.1(−)-CS1 immunized group on days 60 and 75 vs. antibody titer of smegmatis + pDE22-CS1 immunized group on day 60s and 75.
Groups of mice were vaccinated with recombinant \emph{M. smegmatis}, DNA vaccines or controls, and their splenic T cell response to the CS1 antigen was characterized by MTT assay and LDH-based cytotoxicity analysis. After CS1 stimulation, splenic cells from the mice vaccinated with pcDNA3.1(−)-CS1 proliferated poorly, which was similar to those from control mice. In contrast, splenic cells from the mice vaccinated with recombinant \emph{M. smegmatis}-pDE22-CS1 responded strongly to the CS1 \textit{ex vivo}, with a significantly higher proliferation index \((p<0.05, \text{Student's } t\text{-test})\) compared with other groups (Fig. 4a). Interestingly, splenic cells from the mice injected with pcDNA3.1(−)-CS1 did kill CS1-expressing P815 cells \textit{ex vivo}, and their CS1-specific CTL activities increased in parallel with the ratios of effector to target E:T (Fig. 4b). Remarkably, splenic cells from the mice vaccinated with recombinant \emph{M. smegmatis}-pDE22-CS1 killed specifically about 50.2\% CS1-expressing P815 target cells, which was significantly higher than that of the cells from DNA-vaccinated mice \((p<0.05, \text{Student’s } t\text{-test})\). Accordingly, these observations indicate that the vaccination of mice with recombinant \emph{M. smegmatis} not only induces strong antigen-specific T cell proliferation, but also promotes high levels of CTL responses.

4. Discussion

Progress has been made in the development and application of hepatitis B vaccines produced in yeast or mammalian cells for protecting human from HBV infection worldwide (Bo et al., 2005; Diminsky et al., 1999). However, HBV infection and its serious consequences are still a major concern for global public health, especially in Eastern Asia including China. The failure of the classical HBV vaccines in a small population of individuals to induce protective immune responses is complicated by inoculation. However, the mice vaccinated with DNA or the control did not show any abnormal behavior or injury.

Importantly, the mice vaccinated with recombinant \emph{M. smegmatis}-pDE22-CS1 or pcDNA3.1(−)-CS1, but not controls, developed high titters of antibodies against the purified CS1 antigen. Interestingly, a strong humoral response (i.e. the level of antibodies) induced by CS1-based DNA vaccination appeared shortly after immunization, reached a peak about 45 days post-immunization, and then declined (Table 1). In contrast, the humoral response in the mice vaccinated with recombinant \emph{M. smegmatis}-pDE22-CS1 was significantly lower than that in the mice injected with DNA vaccine \((p<0.05, \text{ANOVA})\) 15 days post-immunization. However, the antibody level continued to increase up to 60 days post-immunization which maintained at a higher level for a longer period of time, compared with that in the mice injected with DNA vaccine \((p<0.05, \text{ANOVA})\). These data suggest that although DNA vaccination can induce rapidly and effectively antigen-specific antibodies for a shorter period vaccination with recombinant \emph{M. smegmatis} stimulates a longer period of humoral response as compared with that of the DNA vaccine \textit{in vivo}.  

3.6. T cell responses to the CS1
many factors, such as the improper storage or administration of the vaccine, age, sex, obesity and immunosuppression, and genetically determined resistance (Godkin et al., 2005; Shouval et al., 1994).

HBcAg, highly conserved, can be divided into two domains. The domain covering N-terminal 150 amino acids is required for its oligomerization into capsids, whilst the other covering the carboxyl terminal 34–36 amino acid is an arginine-rich region that non-specifically binds to nucleic acids (Le Pogam et al., 2005). The truncated HBcAg sequence 1–150 has been expressed efficiently in prokaryotic cells (Kratz et al., 1999). HBcAg is strongly immunogenic because it contains potent cytotoxic determinants, especially for peptide 18–27 presented by HLA-A0102 (Kakimi et al., 2002; Townsend et al., 1997).

Furthermore, during chronic HBV infection, pro-inflammatory T cell immune responses to HBcAg are primed preferentially (Beckebaum et al., 2003; Milich et al., 1997). Hence, HBcAg can be used as one of the antigens for the development of vaccines. In addition, the highly conserved preS1 protein, which is responsible for the binding of virus to hepatocytes, is capable of stimulating neutralization antibodies in vivo and is important for the generation of new vaccines against HBV (Jones et al., 1999; Madalinski et al., 2004; Paran et al., 2001). Thus, the generation of recombinant DNA or live bacterial vaccines to express both core and preS1 antigens probably induces antigen-specific immune responses, prevents HBV infection. In the present study, a recombinant DNA vaccine and a recombinant senegatis mycobacterium were generated successfully. Unlike the HBsAg gene, which is difficult to express in prokaryotic cells (Yang et al., 2003), the fused genes of the truncated core and preS1 could be expressed in both prokaryotic and eukaryotic cells. The addition of preS1 peptide to the carboxyl terminus of the truncated HBcAg sequence 1–155 did not affect the antigenicity and immunogenicity of HBcAg, which was consistent with the results of a previous report (Chen et al., 2004). Thus, the CS1 antigen produced in either prokaryotic or eukaryotic cells can be used as an antigen for the detection of both anti-HBc and anti-preS1 antibodies. Indeed, most anti-HBc or anti-preS1 positive human sera were identified by ELISA using the purified CS1 protein although their specific subclasses remain to be determined. Given that the majority of Chinese patients with hepatitis B already progressed to chronic hepatitis and their sera contain mainly HBV-specific IgG, the antibody detected against CS1 was likely to be predominant by IgG of this subclass.

DNA-based vaccines are cheaper to produce and easier to administer. However, DNA vaccines usually induce a shorter period of gene expression and poorer antigen-specific immune responses in vivo since the injected DNA is sensitive to metabolic degradation mediated by endogenous enzymes (Condon et al., 1996). Transfection with the CS1-expressing plasmid induced stable expression of CS1 protein in vitro. However, CS1-specific DNA vaccination only induced a short peak of humoral responses and lower levels of T cell immunity. These findings, together with the fact that a large amount of DNA is required for vaccination, suggest that DNA vaccines, at least for the CS1 antigen, may have little value for the control of hepatitis B.

Indeed, the results from numerous DNA-vaccine-based studies are disappointing even when the DNA vaccines were administered in combination with CpG, interleukins, or hot shock proteins (Chow et al., 1997; H.T. Li et al., 2005; X. Li et al., 2005; Malanchere-Bres et al., 2001; Peng et al., 2003).

Mycobacterium has been used to generate recombinant live vaccines (Rezende et al., 2005; Uno-Furuta et al., 2003). Modified BCG has been used successfully to deliver antigens of HIV, streptococcus, spirochete, and other microorganisms or protozoa (Aldovini and Young, 1991; Langermann et al., 1994; Supply et al., 1999). However, it is difficult to produce BCG due to its slow growth cycle. M. smegmatis is non-pathogenic which grows rapidly in vitro (Averill et al., 1993; Dietrich et al., 2003; Eremeev et al., 1996). Live recombinant M. smegmatis can multiply and survive for a long time, leading to the continuous expression of the designed antigen in vivo. Recombinant M. smegmatis, which expressed the CS1 antigen in vitro, induced longer period of humoral responses to the target CS1 antigen than that of DNA vaccination in vivo. Antibodies against the peptide of preS1, especially from amino acid 21 to amino acid 47 have been demonstrated to neutralize effectively the ability of HBV to bind to human hepatocytes (Jones et al., 1999; Kratz et al., 1999; Paran et al., 2001), and the core antigen-based immunity can eliminate infected HBV in vivo. Importantly, vaccination with the recombinant M. smegmatis promoted strong CS1-specific T cell proliferation and CTL responses, which are crucial for virus-specific vaccines (H.T. Li et al., 2005; X. Li et al., 2005). Because NK, NKT cells, and activated macrophages can kill P815 cells in a CD1-dependent or antigen-non-specific fashion they may also contributed to the observed cytotoxicity of P815 cells. Together, the quality and quantity of the immune responses induced suggest that the recombinant M. smegmatis may be applied for the prevention of HBV infection and intervention of hepatitis B in clinical practice.

In conclusion, a recombinant DNA vaccine and a live recombinant M. smegmatis vaccine were produced successfully and they expressed the target CS1 antigen with excellent antigenicity. Vaccination of mice with the recombinant M. smegmatis induced a stronger and longer period of humoral and T cell responses compared with that DNA vaccine. The quality and quantity of CS1-based immune responses suggest that the recombinant M. smegmatis is a valuable candidate vaccine for the prevention of HBV infection. Potentially, the strategy for the generation of recombinant M. smegmatis may aid in the design of new vaccines for the prevention of other viruses-mediated diseases in humans.

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