



Expression of hepatitis B virus surface antigen determinants in *Lactococcus lactis* for oral vaccination

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Received 12 January 2010; received in revised form 3 February 2010; accepted 6 February 2010

KEYWORDS

Lactococcus lactis;
HBV;
Oral immunization;
Interferon;
Adjuvant

Summary

Lactococcus lactis with non-pathogenic and non-colonizing properties is an attractive candidate for delivering biologically active proteins by mucosal routes. In this report we described recombinant *L. lactis* applicable for the development of live mucosal vaccine against hepatitis B virus (HBV). The *PreS* region of the HBV surface antigen alone or combined with “a” determinant of *S* region (*PreSa*) was cloned and expressed in the food grade bacterium *L. lactis* using a nisin-controlled expression (NICE) system. Western blot analysis indicated that both *PreS* and *PreSa* fusion proteins were successfully expressed in *L. lactis* after nisin induction. Oral immunization of BALB/c mice with *PreS* and *PreSa*-producing strains induced both mucosal (intestinal IgA) and systemic (serum IgG) immune responses against HBV at the same magnitude. Two additional groups of mice given *L. lactis* expressing human interferon-alpha 2b as an adjuvant with the *PreS* or *PreSa*-producing strains produced higher IgG but not IgA antibody responses. These results indicated that the lactococci-derived vaccines could be promising candidates as alternative HBV vaccines for preventing hepatitis B.

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Introduction

Hepatitis B virus (HBV) infection is a serious threat to human health worldwide, and causes significant morbidity and mortality in chronic carriers of HBV. Despite the availability of a commercially used yeast-derived HBV vaccine,

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around 5-10% of the vaccinees do not develop protective immunity against the virus (Valenzuela et al., 1982). Additionally, the necessity of intravenous administration and high cost limit its use for large populations in developing countries. Therefore, an effective, inexpensive and easily administered vaccine needs to be developed.

By using *Lactococcus lactis* as an antigen delivery vehicle, the problems described above could be potentially circumvented. *L. lactis* is a gram-positive bacterium with "GRAS" (generally regarded as safe) status, which has been used in fermentation and preservation of food for decades (Nouaille et al., 2003). These food-grade bacteria do not produce lipopolysaccharides or other toxins, and their recombinant products do not require purification. They are also attractive candidates for delivering bioactive proteins through mucosal routes because of their non-pathogenic and non-colonizing properties as well as their abilities to induce immune responses effectively (Wells et al., 1996; Robinson et al., 1997; Pei et al., 2005; Hou et al., 2007; Scavone et al., 2007). To date, a number of antigens of bacterial or viral origin have been produced by *L. lactis* in a form that can be presented to and processed by the immune system of the mammalian host (Le Loir et al., 2005). When the recombinant strains were administered to mice, immune responses could be elicited against these antigens (Xin et al., 2003; Bermudez-Humaran et al., 2004). In the case of mice immunized with *L. lactis* expressing tetanus toxin (TTFC), these responses proved to be protective against lethal challenge with TTFC (Wells et al., 1993; Norton et al., 1997).

The hepatitis B virus surface antigen (HBsAg) is localized to the envelope of HBV, which contains three antigenic domains designated as S, PreS2 and PreS1 (Heermann et al., 1984). The S antigen of HBsAg has been studied in detail and used as a vaccine antigen to prevent infection. The "a" determinant in the S region is common to all four major serological types (adr, adw, ayr and ayw) (Tiollais et al., 1985) of HBV, and can induce neutralizing antibodies against HBV (Waters et al., 1991). When the conformation of the "a" determinant is destroyed by reduction or alkylation, HBsAg loses antigenicity (Mishiro et al., 1980). The PreS1 and PreS2 regions together are known as the PreS domain. Anti-serum against synthetic PreS1 peptide (amino acid 21-47) or PreS2 was able to neutralize the virus and could protect chimpanzees from HBV infection (Itoh et al., 1986; Neurath et al., 1989). In addition, it has been shown that inclusion of the PreS region in a vaccine containing the S region can augmented anti-HBs responses in mice (Shouval et al., 1994).

Many mucosal adjuvants like cholera toxin (CT) or cytokines are required to be co-delivered with vaccines in order to enhance the immune responses (Singh and O'Hagan, 1999). Interferon-alpha (IFN- α) is a cytokine with antiviral, antitumor and immunomodulatory properties. IFN- α has been shown to promote Th1 cell differentiation (Bracci et al., 2008) and is a powerful mucosal adjuvant when administered to mice with human influenza vaccine (Bracci et al., 2005).

In this report, the PreS region of HBsAg as well as the fusion protein including PreS and "a" determinant (PreSa) were expressed in the food grade bacterium *L. lactis* and tested as a candidate vaccine against HBV. At the same time, recombinant *L. lactis* producing human interferon alpha 2b (IFN) was tested as an adjuvant for this vaccine. Our results indicated that serum IgG and intestine IgA were effectively induced in mice orally administered with these recombinant strains.

Materials and methods

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium with aeration at 37 °C, while *L. lactis* strains were cultivated in GM17 at 30 °C without shaking. When necessary, antibiotics and inducers were added as follows: for *E. coli*, ampicillin (100 μ g/ml), chloramphenicol (10 μ g/ml) and isopropyl thiogalactose (IPTG; 1 mM); and for *L. lactis*, Chloramphenicol (5 μ g/ml) and nisin (10 ng/ml).

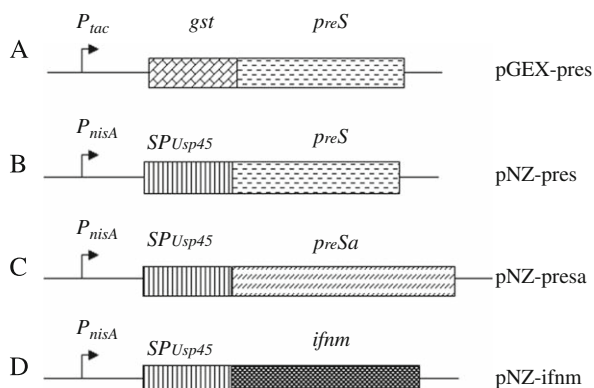
Expression and purification of PreS protein in *E. coli*

For expression and purification of the PreS protein in *E. coli*, the plasmid pGEX-pres was constructed as follows. A 521 bp DNA fragment (nucleotides 1-521), encoding the PreS1 and PreS2 regions, was amplified from plasmid pCMV-LS (containing the full-length HBsAg type adr coding sequence, kindly provided by Professor Yumei Wen, Fudan University) and digested with *Bam*HI and *Xho*I. The purified fragment was then ligated to the same digested pGEX-6p-1 to obtain expression vector pGEX-pres (Figure 1A).

After transformation of *E. coli* BL21 (DE3) with the plasmid pGEX-pres, the recombinant strain was cultured until the mid-exponential phase

Table 1. Bacteria strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Source or reference
Strains		
<i>E. coli</i> JM109	Host for gene cloning	Invitrogen
<i>E. coli</i> BL21 (DE3)	Host for gene expression	Novagen
<i>L. lactis</i> MG1363	Plasmid-free strain	Gasson (1983)
<i>L. lactis</i> NZ9000	Derivative of MG1363 carrying regulatory genes <i>nisR</i> and <i>nisK</i>	Kuipers et al. (1998)
Plasmids		
pUC57	Ap ^r ; <i>E. coli</i> cloning vector	Our lab preserved
pGEX-6p-1	Ap ^r ; <i>E. coli</i> expression vector	This work
pCMV-LS	Kan ^r ; pCMV plasmid with cloned full length HBsAg type adr coding sequence	A gift from Prof. Yumei Wen, Fudan University
pNZ8048	Cm ^r ; <i>L. lactis</i> expression vector; <i>P_{nisA}</i> promoter	Kuipers et al. (1998)
pGEX-pres	Ap ^r ; pGEX-6p-1:: <i>pres</i>	This work
pUC- <i>usp</i>	Ap ^r ; pUC57:: <i>SP_{Usp45}</i>	This work
pUC- <i>usp-pres</i>	Ap ^r ; pUC57:: <i>SP_{Usp45}::pres</i>	This work
pUC- <i>usp-presa</i>	Ap ^r ; pUC57:: <i>SP_{Usp45}::presa</i>	This work
pUC57- <i>ifnm</i>	Ap ^r ; pUC57:: <i>ifnm</i>	This work
pUC45- <i>ifnm</i>	Ap ^r ; pUC57:: <i>SP_{Usp45}::ifnm</i>	This work
pNZ-pres	Cm ^r ; pNZ8048:: <i>SP_{Usp45}::pres</i>	This work
pNZ-presa	Cm ^r ; pNZ8048:: <i>SP_{Usp45}::presa</i>	This work
pNZ- <i>ifnm</i>	Cm ^r ; pNZ8048:: <i>SP_{Usp45}::ifnm</i>	This work

**Figure 1.** Schematic diagram of plasmid constructs carrying *ifnm* and HBsAg fragments. A, pGEX-pres; B, pNZ-pres; C, pNZ-presa; D, pNZ-ifnm. Detailed information on these expression plasmids is given in text and Table 1.

($OD_{600}=0.5-0.6$) and induced with IPTG (1 mM) for additional 5 h. Cells were collected and then resuspended in PBS buffer and sonicated for 5 min until lysed. The inclusion bodies were collected and loaded on 12% SDS-PAGE. After electrophoresis, the gel was washed with ddH₂O and incubated in ice cold 0.3 M KCl until the PreS protein band was visualized. The band was then excised carefully out of the gel, crushed to pieces and incubated in adequate PBS at 4 °C overnight. After centrifugation, the supernatant was carefully removed into a new microcentrifuge tube. The presence of the

protein in the supernatant was verified by SDS-PAGE, and protein concentration was estimated by the bicinchoninic acid assay (Pierce) using bovine serum albumin (BSA) as the standard.

Construction of *L. lactis* expression vectors carrying the *preS* and *preSa* genes

For expression and secretion of the PreS and PreSa protein in *L. lactis*, the gene *SP_{Usp45}* (signal peptide of Usp45, which is a dominant secreted protein in *L. lactis*) was amplified from the chromosomal DNA of *L. lactis* MG1363. It was then inserted into plasmid pUC57 with *EcoRI* and *SacI* restriction sites, getting the plasmid pUC-*usp*.

To construct the plasmid carrying the *preS* gene, the 521 bp *preS* (nucleotides 1-521) fragment was amplified from pCMV-LS and was cloned downstream of *SP_{Usp45}* in pUC-*usp* with *SacI* and *KpnI* to get plasmid pUC-*usp-pres*. The fusion fragment *usp-pres* was then cloned into the pNZ8048 skeleton with *NcoI* and *KpnI* restriction sites, generating the plasmid pNZ-pres (Figure 1B).

Similarly, to construct the PreSa expression vector, the “a” (nucleotides 816-1032) fragment was fused to the 3' end of the *preS* gene by PCR and the resulting *preSa* fusion fragment was inserted into pUC-*usp* at *SacI* and *KpnI* sites to get plasmid pUC-*usp-presa*. The 840 bp *usp-presa* fragment was

amplified from pUC-*usp-presa*, digested by *Nco*I and *kpn*I restriction sites and ligated to the same digested pNZ8048, yielding the expression plasmid pNZ-*presa* (Figure 1C).

Expression of PreS and PreSa proteins in *L. lactis*

The inducible vectors pNZ-*pres* and pNZ-*presa* were introduced into *L. lactis* NZ9000 by electroporation as described previously (Holo and Nes, 1989). *L. lactis* strains were grown to an OD_{600} of 0.5-0.6 and followed by induction with 10 ng/ml nisin (Sigma-Aldrich) for 6 h. Protein extractions were performed as described previously (Le Loir et al., 1998) and separated on a 12% SDS-PAGE gel. The proteins were transferred onto a PVDF (Amersham Pharmacia Biotech, UK) membrane, where the immunoblots were developed using a polyclonal anti-mouse preS2 antibody at a dilution of 1:500 (Beijing Hepatitis Reagent Research and Production Center). Horse-radish peroxidase (HRP)-conjugated anti-mouse IgG (H+L) (Biolinks) secondary antibody diluted at 1:5000 was used for the detection of specific antibody binding. The bands were visualized using enhanced chemiluminescence reagents (Applygen, Beijing, China) according to the manufacturer's instructions.

Production of IFN in *L. lactis*

The native IFN gene was designed according to the codon bias of *L. lactis* (http://gib.genes.nig.ac.jp/single/codon/main.php?spid=LLac_MG1363) and was artificially synthesized by Sino-American Biotech (Luoyang, Henan, China). The resulting 498 bp gene named *ifnm* was ligated into the *Sac*I and *Kpn*I restriction sites in plasmid pUC57, yielding plasmid pUC57-*ifnm*.

To produce and secrete IFN in *L. lactis*, the gene *SP_{Usp45}* was inserted into pUC57-*ifnm* with *Eco*RI and *Sac*I restriction sites, getting the plasmid pUC45-*ifnm*. Then the *usp-ifnm* DNA fragment was amplified, digested by *Nco*I and *Kpn*I enzymes and ligated into a same digested pNZ8048 vector, resulting in plasmid pNZ-*ifnm* (Figure 1D).

The expression of IFN in *L. lactis* NZ9000 transformed with plasmid pNZ-*ifnm* was detected by Western blot analysis using a monoclonal anti-mouse interferon alpha 2b (Abcam, ab9386) at a dilution of 1:1000 as the primary antibody.

Mouse vaccination

Eight-week old specific pathogen free female BALB/c mice were purchased from the Animal Research Center of the Medical Department in Peking

University (Beijing, China). Five groups of mice (seven or eight mice in each group) were orally immunized with negative control and recombinant strains on days 0, 1, 2; 14-16 and 28-30. Two groups of mice were immunized with 2×10^9 colony-forming units (CFUs) of *L. lactis* NZ9000 strains containing plasmids pNZ-*pres* (NZS) and pNZ-*presa* (NZSA) in 0.1 ml sterile PBS, respectively. The negative control of mice received wild type *L. lactis* NZ9000 (NZ). To enhance the immune responses in mice, *L. lactis* NZ9000 carrying plasmid pNZ-*ifnm* (NZI) was used as an adjuvant. For two other groups of mice, one group was given 10^9 CFU of NZS and 10^9 CFU of NZI (NZSI), and the other group received 10^9 CFU of NZSA and 10^9 CFU of NZI (NZSAI). Plate counts were performed with all inoculums to corroborate the CFU administered.

Sample collection

Serum samples were collected on days 21, 35 and 51 and drawn from the orbital plexus of each mouse. After clotting and centrifugation, serum samples were collected and stored at -20°C until tested.

Intestinal secretions were obtained as follows. After the mice were sacrificed at day 51, the small intestine of each mouse was collected. The contents in the intestine were gently removed by washing with PBS. The intestinal secretions were then harvested by flushing the lumen with PBS and 1 mM phenylmethanesulfonyl fluoride (PMSF). Mouse intestinal contents were centrifuged and the supernatants (gut washings) were stored at -20°C until assayed.

Antibody ELISA

ELISA plates (Costar, Corning Incorporated, NY, USA) were coated with 0.5 μg purified GST-PreS protein in 100 μl 0.1 M sodium carbonate buffer (pH 9.5) per well overnight at 4°C . Blocking with PBS containing 0.05% Tween 20 (PBST) and 5% skim milk was performed at 37°C for 2 h. Serum samples (100 μl per well) were added in duplicate at the appropriate dilution (1:5) as primary antibodies. For the detection of intestine IgA, the supernatants of the extracts were diluted at 1:10 in the same manner as those used for serum specimens. The plates were then incubated for 2 h at 37°C following six washes with PBST. Bound antibodies were detected using HRP-conjugated goat anti-mouse IgG (Biolinks, 1:5000 dilution) or IgA (Sigma, 1:5000 dilution). After incubation at 37°C for 1 h, the plate was washed with PBST six times, and developed with

100 μ l TMB (3,3',5,5'-tetramethylbenzidine) as substrate. Absorbance was then measured at 450 nm.

Statistical analysis

The antibody responses of immunized mice were measured as absorbance values (OD) in ELISA. Statistical significance was determined by Student's *t*-test, where $P < 0.05$ was considered to be statistically significant.

Results

Expression and purification of PreS protein in *E. coli*

The *PreS* gene was cloned and expressed in *E. coli* BL21 (DE3) using plasmid pGEX-pres. The resulting protein GST-PreS was used as a positive control in Western blotting and a coating antigen in antibody ELISA assay. After induction with different concentrations of IPTG, the protein samples were prepared and subjected to SDS-PAGE. As shown in Figure 2, the expression of protein GST-PreS was increased with increasing concentration of IPTG used. The expression level reached the highest when 1 mM IPTG was applied. But most of the GST-PreS proteins at the expected size of 44 kDa were forming inclusion bodies rather than soluble forms. The GST-PreS proteins were then purified from the gel as described above.

Expression of HBsAg fragments in *L. lactis*

The PreS and PreSa regions of HBsAg were selected to be expressed in *L. lactis* NZ9000. After induction with nisin, the expressions of PreS and

PreSa fusion proteins in NZS or NZSA were analyzed by immunoblotting using an anti-mouse PreS2 antibody. Purified GST-PreS protein was included as a positive control, while NZV was used as a negative control. As shown in Figure 3, specific signals were detected in the cellular fractions of the two recombinant strains at expected sizes of 21 and 29 kDa, which correspond to the precursor forms of PreS and PreSa from NZS and NZSA, respectively. A slight degradation of PreSa protein was observed in the NZSA sample. There was no PreS signal detected in the supernatant fractions of

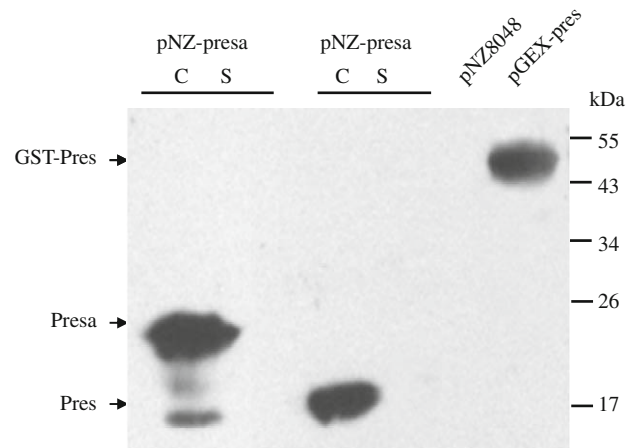


Figure 3. Expression of PreS and PreSa proteins in *L. lactis*. When the recombinant *L. lactis* culture reached an optical density of 0.6 at 600 nm, 10 ng/ml nisin was added to induce the expression of PreS and PreSa for 3 h. Cell extracts and culture supernatants were then prepared and analyzed by immunoblot using a polyclonal anti-mouse preS2 antibody. Purified GST-PreS was used as a positive control and cell extract of *L. lactis* expressing pNZ8048 as a negative control. Sizes (kDa) of the prestained marker proteins are shown on the right.

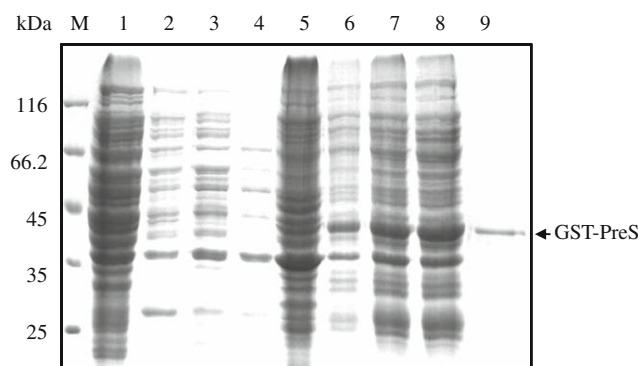


Figure 2. Expression and purification of PreS protein in *E. coli*. Protein extracts from different concentrations of IPTG induced *E. coli*/pGEX-pres strain (lanes 1-8) were separated by 12% SDS-PAGE. Lane 1-4, soluble fractions of *E. coli*/pGEX-pres strain induced by 0, 0.25, 0.5 and 1 mM IPTG; lane 5-8, inclusion bodies of *E. coli*/pGEX-pres strain induced by 0, 0.25, 0.5 and 1 mM IPTG. The purified GST-PreS was loaded in lane 9. Sizes (kDa) of the molecular mass marker (M) are indicated on the left.

induced NZS or NZSA samples, likely due to the low secretion efficiencies of PreS and PreSa proteins. As expected, no signal was found in either cellular or supernatant fractions of the control strain NZV. Hence, the results indicate that the recombinant *L. lactis* strains were able to express the PreS or PreSa proteins without compromising its antigenic properties.

Expression and secretion of IFN in *L. lactis*

To test if IFN could enhance the immune responses in mice immunized with PreS and PreSa-producing *L. lactis* strains, the gene *ifn* was also expressed in *L. lactis*. The native *ifn* gene without its secretory signal peptide was initially expressed. However, no band was observed by Western blotting probably due to low or non-expression of IFN (data not shown). In order to increase the expression level of IFN in *L. lactis*, a new gene *ifnm* was designed and synthesized according to the codon usage of *L. lactis* without changing the protein sequence. The *ifnm* gene was placed under the P_{nisA} promoter, and signal peptide SP_{Usp45} was employed to secrete IFN out of the cells.

Recombinant NZI was examined for the production and secretion of IFN in *L. lactis*, and a negative control NZV was used at the same time. After induction with nisin, protein samples were analyzed by SDS-PAGE and Western blot assays. Induced protein band was clearly visible in the cellular fraction of NZI with the expected size of 22 kDa on SDS-PAGE, which corresponds to the precursor form

of Usp45-IFN (Figure 4A). The expression of IFN was further demonstrated by Western blotting using a mouse monoclonal antibody to interferon α -2b (Abcam, ab9386), and IFN signals were detected in both cellular (C) and supernatant (S) fraction of NZI. Figure 4B shows that IFN was successfully secreted by the signal peptide SP_{Usp45} , although the production level of IFN in the supernatant was lower than that in the cellular fraction.

Immune responses induced by intragastric immunization

Forty mice were randomly divided into five groups and the eight mice in each group were administered orally with control strain *L. lactis* NZ9000 (NZ) and recombinant strains. The local responses in the intestinal samples were examined by ELISA using purified GST-PreS protein as the coating antigen. Results indicated that mice orally administered with NZS and NZSA strains induced very significant antigen-specific mucosal IgA responses compared with the control group of mice, which received native NZ ($P < 0.05$). However, there was no obvious difference in PreS-specific IgA level between mice immunized with NZS and NZSA strains ($P > 0.05$), whose mean ELISA absorbance values were 0.54 and 0.53 on day 51, respectively (Figure 5). No anti-PreS antibodies were observed in the control group of mice given native NZ.

Likewise, serum PreS-specific IgG from immunized mice were also determined (Figure 6). Both

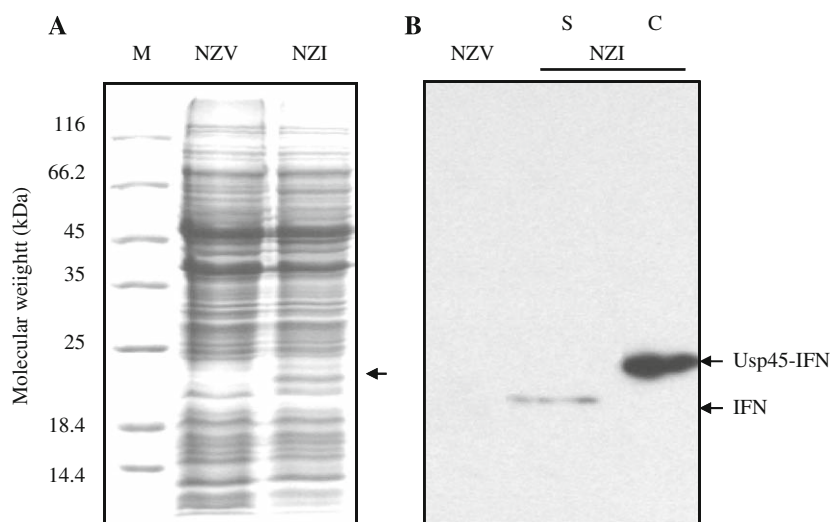


Figure 4. Production of IFN protein in *L. lactis*. After induction with nisin, the IFN protein produced by recombinant strain *L. lactis* NZ9000/pNZ-ifnm (NZI) was extracted and analyzed by SDS-PAGE (A) and Western blotting (B). Cell extracts of *L. lactis* NZ9000/pNZ8048 (NZV) was used as a negative control. Arrows indicate the positions of the Usp45-IFN and IFN proteins. C: cellular fraction; S: supernatant fraction. Sizes (kDa) of the molecular mass marker are indicated on the left.

groups of mice that received NZS and NZSA developed a similar level of PreS-specific serum IgG ($P < 0.05$). The IgG absorbance value of the two groups of mice could be detected ($OD = 0.13$) on day 21, then reached the highest reading ($OD = 0.27$) on day 35 and decreased slightly ($OD = 0.21$) on day 51 (Figure 6). The overall induction of specific serum IgG was weaker than that of mucosal IgA. Taken together, the results showed that the recombinant lactococci generated in this study were able to elicit both HBV specific systemic and mucosal antibody responses on oral administration.

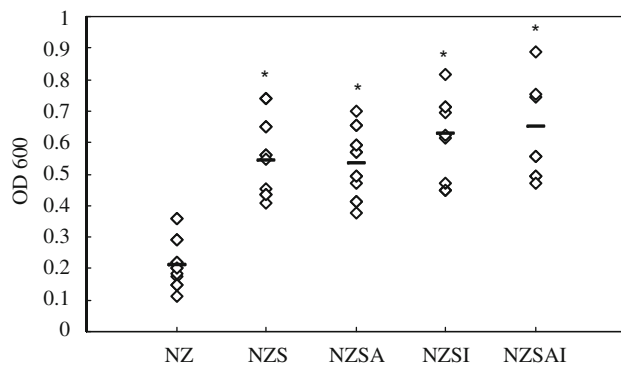


Figure 5. Anti-PreS specific local IgA responses in intestine samples. Groups of mice received three consecutive doses of 2×10^9 CFU recombinant strains, three times at 2-week intervals. The value is determined as the optical density (OD) reading of intestine samples diluted 1:10. Statistically significant differences ($P < 0.05$) are denoted by an asterisk (*) between control and immunized groups.

Influence of co-administration of IFN-producing *L. lactis* strains on immune responses

In order to enhance the immune responses in mice that received recombinant lactococci strains, co-administration of *L. lactis* expressing human interferon alpha (NZI) was given in this study as an adjuvant. The PreS-specific mucosal IgA and systemic IgG antibody responses in groups of mice immunized with NZSI and NZSAI compared with those that received NZS and NZSA were measured to assess the effect of human interferon alpha (IFN) for the recombinant HBV vaccine.

As shown in Figure 5, the mucosal IgA responses were significantly elevated in intestinal samples of groups of mice immunized with strains NZSI and NASAI, producing similar mean absorbance values of 0.63 and 0.65, respectively, in the HBV-specific IgA ELISA ($P < 0.05$). Compared to the two immunized groups of mice receiving NZS and NZSA, the IgA responses were not enhanced obviously in NZSI ($P = 0.13$) and NZSAI ($P = 0.11$) immunized groups.

To determine whether co-administration with the IFN-producing strain would specifically enhance the IgG responses, the PreS-specific IgG in the serum samples was assayed from groups of mice immunized orally with the different strains of *L. lactis*. Strikingly, the anti-PreS antibody titers increased more rapidly and were substantially (~2-fold by day 35) higher in the groups of mice immunized with NZSI ($P = 0.006$) or NZSAI ($P = 0.002$) than in the groups immunized with NZS or NZSA (Figure 6). On day 51 following immunization, the mean PreS antibody responses were still significantly higher in

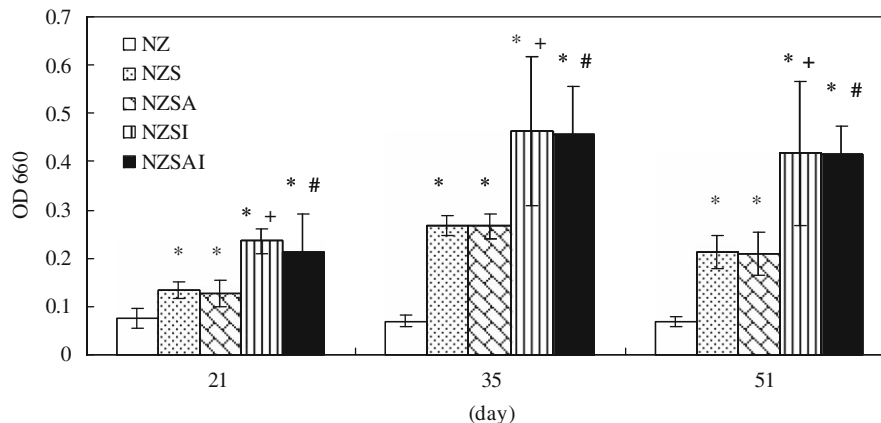


Figure 6. Anti-PreS serum IgG responses induced after oral immunization with recombinant *L. lactis* NZ9000 strains. Sera from groups of mice immunized orally with 2×10^9 CFU *L. lactis* strains were tested for the presence of PreS specific IgG by ELISA, using GST-PreS as the coating antigen. The value is determined as the optical density (OD) reading of serum diluted 1:5. Statistically significant differences ($P < 0.05$) are denoted by an asterisk (*) between control and immunized groups, a cross (+) between NZS and NZSI or by a hash symbol (#) between NZSA and NZSAI immunized groups.

the groups of mice given NZSI ($P=0.008$) or NZSAI ($P = 0.002$) than in the groups that received NZS or NZSA.

Although the enhancement of IgA intensity was not obvious, the IgG responses were significantly augmented in mice co-administered with IFN-producing strains. The results indicated that the lactococci-derived interferon-producing strain could be used as a potential adjuvant.

Discussion

Most pathogenic microorganisms initiate their infectious cycles at the mucosal surfaces, since the large epithelial surfaces such as respiratory, intestinal and urogenital tracts need to be protected against colonization and invasion of infectious agents. This can be achieved by mucosal vaccination, which can not only induce protective immune responses at the mucosa by secretory IgA (McGhee and Mestecky, 1990) but also elicit specific immunity in the systemic lymphoid tissues (Chen, 2000).

In this study, we engineered a food-grade bacterium *L. lactis* to express either the PreS or PreSa protein, and studied the possibility of these recombinant strains as hepatitis B vaccines when orally administered to BALB/c mice. In our animal experiments, anti-PreS IgA antibody responses were detected in intestinal samples at the end of immunization and serum IgG responses in blood on as early as day 21. As HBV is transmitted through contaminated needles and by sexual contact, effective vaccination should induce both systemic and mucosal immune responses (Kumar et al., 2007). These results suggested that our oral vaccine delivery system using the recombinant *L. lactis* is suited for mucosal immunization by inducing both humoral and cellular immune responses.

The selection of antigen is a key factor in developing effective HBV vaccines. Most research programs are focused on attaining high levels of S antigen expression in transgenic plants (Mason et al., 1992; Richter et al., 2000; Kumar et al., 2007) or other microorganisms such as yeast (Schreuder et al., 1996). Meanwhile, the PreS1 and PreS2 surface proteins of HBV were also shown to play an important role in immunogenicity against HBV in mice as well as in humans in a number of comparative and controlled trials (Rendi-Wagner et al., 2006). In the light of the demonstrated role of the PreS1 and PreS2 envelope proteins of HBV in immunogenicity and protective efficacy, in this work we attempted to express the PreS protein in

L. lactis and assessed the immunogenicity of these recombinant strains in mice. As full-length HBsAg in either fusion or non-fusion form could not be produced in sufficient quantities in *E. coli* (Sheu and Lo, 1995), and the “a” determinant of HBsAg could induce virus neutralizing antibodies (Waters et al., 1991), we combined the PreSa fusion protein together to test if they could induce stronger immune responses in mice. The results indicated that mice in groups receiving NZS and NZSA all resulted in production of intestine and serum antibodies against PreS. However, the IgA and IgG antibody responses were almost the same in the two groups of mice. As the “a” determinant contains two specific sulphur bridges (Waters et al., 1991), it is likely that the “a” determinant in the recombinant PreSa protein was not correctly folded as produced by the bacteria in our study.

Immunological adjuvants are substances used in combination with a specific antigen that can enhance immunity over using the antigen alone. The use of cytokines as adjuvants has become a powerful method to enhance immunity elicited by recombinant DNA or protein vaccines (Singh and O’Hagan, 1999). IFN- α is one of the treatments approved for chronic HBV infection. In this study, with mice that received recombinant NZSI and NZSAI orally, IFN- α could indeed increase the magnitude of the IgG response (by nearly 2-fold), indicating its potential as an adjuvant in this model. Yet, the augmentation of IgA responses was not obvious, which might be mainly explained by the low yield of IFN secreted in NZI strain (Figure 4). Considering that purified human IFN- α used as an adjuvant for a human influenza vaccine could not only increase the antigen specific antibody titers but also induce full protection from the virus challenge (Proietti et al., 2002; Bracci et al., 2005), efforts will be made in our further research to enhance the expression and secretion of IFN in *L. lactis*.

In conclusion, in this study we expressed recombinant PreS and PreSa proteins in food-grade bacterium *L. lactis* NZ9000. The expressed PreS and PreSa proteins could induce both systemic and mucosal immune responses against PreS in mice to the same level, implying that this lactococci-derived PreS protein could be developed as an alternative oral vaccine for preventing HBV infection in humans.

Acknowledgments

This work was supported by grants from the National Program for High Technology Research and

Development of China (2006AA10Z319) and the Key Project of the Chinese Academy of Sciences (KSCXZ-YW-G-016). We greatly appreciate Prof. Yumei Wen and Dr. Xin Yao for their kindly presentation of the plasmid pCMV-LS.

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