

Enhancement of mucosal immune response against the M2eHBc+ antigen in mice with the fusion expression products of LTB and M2eHBc+ through mucosal immunization route

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Abstract M2e is the external domain of M2 protein, a conservative transmembrane protein of the avian influenza A virus. Previous research had shown that the vaccine of the formation particle of M2e and hepatitis B virus core antigen (HBcAg) can fully protect mice against a lethal H5N1 subtype avian influenza virus (AIV) infection. As an effective approach against mucosal tissue infectious agent, mucosal vaccination requires effective and safe adjuvants. Here we have first fused two M2e peptide to the N terminal and the major immunodominant region (MIR) of the HBcAg protein simultaneously to create a fusion gene, named as M2eHBc+, and then inserted B subunit of *Escherichia coli* heat labile enterotoxin (LTB) into the N terminal of M2eHBc+ to construct the second fusion gene, named as LBM2eHBc+. These two fusion genes can be efficiently expressed in *Escherichia coli* cell and the yield peptide can self-assemble into virus-like particles (VLP). The mice immunization with two types of the purified particles by intranasal dropping and oral routes revealed that LTB can significantly enhance the mucosal immune responses of mice to co-expression M2eHBc+ particle form antigen.

Keywords Avian influenza virus · HBcAg · M2eHBc+ · LTB · Mucosal immunity

Abbreviations

AIV	avian influenza virus
BSA	bovine serum albumin
CT	cholera toxin
CTB	cholera toxin B subunit
GM1	galactosyl-N-acetylgalactosamylsialyl-galactosylglucosylceramide
HBcAg	hepatitis B core antigen

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IPTG	isopropyl β -D-thiogalactopyranoside
LT	<i>Escherichia coli</i> heat labile enterotoxin
LTB	B subunit of LT
M2e	the extracellular part of the M2 protein
MIR	immunodominant region
PBS	phosphate buffered saline
VLP	virus-like particle

Introduction

In general, avian influenza virus (AIV) only infects avian species. *Homo sapiens* is not the natural host of this type of virus. However, human infections with pathogenic AIV in different countries were incessantly reported since 1997 (Subbarao et al. 1998). Hence, the effectively control of avian influenza has become not only important to the healthy development of livestock and poultry breeding industry, but also critical to prevent a pandemic AIV outbreak in human society. Among the available strategies, vaccination remains the most effective approach for controlling the avian influenza threat. In the meantime, the ongoing variations of the surface glycoprotein of the hemagglutinin and neuraminidase in AIV impose limitations on the vaccine research and development. Vaccines based on hemagglutinin and neuraminidase should be annually updated in order to match the current epidemic strain of the virus. An alternative strategy to bypass this uncertainty could be developing novel vaccines that targeting the conservative surface antigen. M2 protein is the highly conservative protein that constitutes the spike of the envelope of AIV (Ito et al. 1991). Its N terminal carries 24 amino acid residues that exposed to the membrane surface (M2e), which is the induction epitope of the neutralizing antibody in M2 protein. The monoclonal antibody based on M2e can effectively inhibit the replication of influenza virus in the *in vitro* cultured cells and in mice as well (Zebedee and Lamb 1988; Treanor et al. 1990). However, the M2e alone, which contains only 24 amino acid residuals, cannot effectively stimulate the organism's immune responses. Large macromolecules are needed to couple with M2e to elicit effective immune responses (Neirynek et al. 1999; Fan et al. 2004; De Filette et al. 2005, 2006; Ernst et al. 2006). Hepatitis B core antigen (HBcAg), a structural protein of Hepatitis B virus, is one of the widely used epitope vaccine carriers. Previous investigations have indicated that the M2e fusion proteins vaccine, either fusing M2e peptide into the N terminal of HBcAg or integrating into the major immunodominant region (MIR) of HBcAg, can be successfully expressed in prokaryotic cells. Either one of the M2e fusion vaccine was able to fully protect mice against a lethal virus challenge (Neirynek et al. 1999; De Filette et al. 2005, 2006).

Mucosal system is the major barrier separating the organism to the infectious agents. Therefore, vast amount of the infections occur at or emanate from mucosal surfaces, such as *Vibrio cholerae*, enterotoxigenic *Escherichia coli* caused gastrointestinal tract infections, AIV, foot and mouth disease virus caused respiratory tract infections, etc. Immunization through mucosal pathway that directly aiming at these pathogenic agents would be an efficient anti-pathogen strategy, as the secretory IgA (sIgA) secreted by the mucosal immune system after the stimulation from the antigen can prevent the infectious agent from attaching and colonizing in the mucosal epithelium (Eriksson and Holmgren 2002). However, only a few vaccines can stimulate the immune responses of mucosal system through mucosal immunization pathway. Most of the vaccines need mucosal adjuvant to effectively elicit the immune response (Lauterslager and Hilgers 2002). Among the known

immunoadjuvants, heat-labile enterotoxin (LT) from *Escherichia coli* and its close homologue cholera toxin (CT) from *Vibrio cholerae* are two of the most potent. Unfortunately their intrinsic toxicity limited their clinical applications. Many attempts have been made to detoxify LT or CT while trying to preserve their adjuvant activity. Several non-toxic mutants of LT/CT and non-toxic B subunits have been studied in experimental mucosal immunization (Dickinson and Clements 1995; Verweij et al. 1998; Williams 2000; De Haan et al. 2001; Richards et al. 2001; Millar et al. 2001). Given the advantages of mucosal immunization against the AIV invasions, the inventing novel and safe mucosal immunization and mucosal pattern vaccines is among the most perspective studies for avian influenza vaccines research (Verweij et al. 1998; Tumpey et al. 2001; De Haan et al. 2001; De Filette et al. 2006). B subunit of LT (LTB), the non-toxic and highly active mucosal adjuvant, has been widely used in the mucosal vaccine development (Richards et al. 2001; Millar et al. 2001; Verweij et al. 1998; De Haan et al. 2001; Yamanaka et al. 2006; Fingerut et al. 2006; Companjen et al. 2006).

In this study, two M2e epitopes have been simultaneously fused to the N terminal and MIR of the full-length HBcAg to construct the prokaryotic expression vector pM-M2eHBc+. Further, LTB has been inserted into the N terminal of M2eHBc+ to create the expression vector pM-LBM2eHBc+, which is able to co-express LTB and M2eHBc+ protein. Both types of the fused genes can express soluble protein in *Escherichia coli* cell, and these two kinds of protein was used to immunize mice by intranasal dropping and oral routes, and the results showed that LTB can significantly enhance the mucosal immune responses of mice to co-expression M2eHBc+ antigen.

Materials and methods

The construction of pM-M2eHBc+ and pM-LBM2eHBc+ expression vectors

pMD-LT plasmid (genbank accession number EF057802), pET32a-M2 plasmid, 1301-HBcAg plasmid were stored by our laboratory.

M2eHBc+ coding cDNA was amplified by over-lap polymerase chain reaction (PCR) according to Frace et al. (1999). A total of 8 primers (Table 1) were synthesized. Note there are 18-nt complementary sequence in M2eR and M2eHF, and 15-nt complementary sequence in M2eH1R and M2eH2F. Both M2eF and M2eHR primers contain one

Table 1 Primers used in this work

Name	Sequence
M2eF	5'-GGCGTCGACATGAGTCTTCTAACCGAGG-3'
M2eR	5'-GTCAATGTCAGGATCACTTGAATCGCTG-3'
M2eHF	5'-AGTGATCCTGACATTGACCCGTATAAAG-3'
M2eH1R	5'-CCAACCGTTACGGGTTGGGTTTCCACTTCGGTCAGCAGGCTTGCTGGG TCTTCCAAATTAC-3'
M2eH2F	5'-ACCCGTAACGGTTGGGAATGCCGTTGACGCGATAGCAGCGATTCCAGGGA ATTAGTAGTCAG-3'
M2eHR	5'-GGCAAGCTTCTAACATTGAGATTCCCGAG-3'
LTB-F	5'-GGCGAATTCATGAATAAAGTAAATG-3'
LTB-R	5'-GGCGGATCCCGGGCCGTTTTCCATACTGATTG-3'

endonuclease cutting site, which is *SalI* and *HindIII* respectively to facilitate the insertion of the PCR amplified fragment into the prokaryotic expression vector pMAL-c2x (New England Biolabs, NEB). Similarly, LTB-F and LTB-R contain one endonuclease site, which is *EcoRI* and *BamHI* respectively to facilitate the insertion of LTB into the N terminal of M2eHBc+.

The induced expression of the target genes and the purification of products

The sequence of pM-M2eHBc+ and pM-LBM2eHBc+ was confirmed by sequencing. Plasmids were used to transform the competent cell, Rosetta (DE3) strain (Novagen). The positive clones were inoculated in the Luria-Bertani broth containing 50 mg/l chloromycetin and 50 mg/l ampicillin antibiotics. The bacterial was sub-cultured at 37°C till to OD600 reached to 0.8, then 0.8 mg/l IPTG was added to induce the expression of the fused protein. After the additional induction of 4 h at 37°C, the bacteria cells were collected. The expression products were monitored by running a SDS-PAGE. The protein purification was carried out following the methods described in Ng et al. (2006) with minor modifications. In detail, the bacteria cells were resuspended with phosphate buffered saline (PBS), and then subjected to ultrasonic fragmentation. The sonicated debris was incubated at 37°C for 8 h and then at 60°C for another 45 min. After 30 min centrifugation at 12000 rpm, the target protein existed in the supernatant was purified by using an amylose resin system (NEB) under native conditions as recommended in the manufacturer's protocol. The tag protein in the pMal-c2x vector, a maltose-binding protein, was removed by Factor Xa excision. The purification of the target protein without the tag protein was carried out by employing the pMAL Protein Fusion and Purification System (NEB). Finally the purified target protein without maltose-binding protein tag was subjected to a thorough dialysis process with PBS at 4°C and condensed using freezing drying method.

The antigenicity analysis and GM1-ELISA detection for the expression product

The purified M2eHBc+, LBM2eHBc+ protein and the negative control were separated by SDS-PAGE, and then transferred to the nylon membrane. Western blotting analysis was carried out according to Sambrook et al. (1989), using rabbit anti-H5N1-M2 antibody (Beijing Biosynthesis) as primary antibody and horseradish peroxidase labeled goat anti-rabbit IgG as secondary antibody (Huamei biology).

Ligand-binding activity of LBM2eHBc+ was examined using GM1-ELISA. 100 µl of 3 µg/ml of ganglioside Galactosyl-N-acetylgalactosamylsialyl-galactosylglucosylceramide (GM1) (Sigma) in PBST buffer (1 l PBS buffer containing 500 µl Tween-20) was utilized to coat the ELISA plate. The distilled water and 5% bovine serum albumin (BSA) solution were used as the controls with triplet repeats. After coating the plate overnight at 4°C, the coating substance was removed. The plate was then blocked with the blocking solution (5% BSA in PBST buffer) at 37°C for 1 h. The coating solution was removed from the plate. The plate was rinsed with PBST three times (this rinsing step was repeated every time before replacing new solution to the plate). 100 µl of purified M2eHBc+ and LBM2eHBc+ with the concentration of 10 µg/ml each, was added into the plate wells while the recombinant cholera toxin B subunit (CTB) (Zhang et al. 2006) of the same concentration was used as the positive control. The plate was incubated at 37°C for 1 h. Replace the buffer with the rabbit anti-CT serum (Sigma, diluted with the blocking solution in 1 : 4000) for 1 h incubation at 37°C and then replace the serum with the secondary antibody, horseradish peroxidase labeled goat anti-rabbit IgG (Huamei biology, 1 : 4000 dilution) for

another 1 h incubation. Remove the antibody from the plate. Briefly (5 min) incubate the plate with 100 μ l tetramethylbenzidine substrate. Finally, the reaction was stopped by addition of 50 μ l of stop buffer (2 M sulphuric acid solution). Measure the absorbance at 450 nm for each well.

Observations by transmission electron microscopy for the fusion protein M2eHBc+ and LBM2eHBc+ expressed in prokaryotic cells

10 μ l purified M2eHBc+ and LBM2eHBc+ fusion protein were loaded on a carbon membrane on top of a copper screen. After staining the sample with 2% phosphotungstic acid for 5 min, air dry the sample for 2 h. The sample was examined with JEM2100 transmission electron microscope to observe whether the two types of expression products can self-assemble into virus-like particles (VLP).

Animal immunization and the collection of serum and small intestine lavage fluid

30 female Balb/c mice of 6-week-old were divided evenly into two groups, corresponding to the two immunization routes, namely intranasal (i.n.) and oral immunization route (o). Within each immunization route, each group was further divided into three sub-groups (1, 2 and 3) with five mice each. The first group was immunized with PBS; the second group was immunized with the purified M2eHBc+ protein; and the third group was immunized with the purified LBM2eHBc+ protein. The dosage for intranasal immunization was 20 μ g of each protein for each mouse, while the dosage for oral immunization was 50 μ g of each protein for each mouse. For intranasal dropping immunization, the protein or PBS buffer was dropped into the nasal cavity and made to naturally flow into the body, while the mice were anesthetized with ether. For the oral immunization, the mice were fasted 8 h (water was normally supplied) before infusing protein or PBS into the stomach. Both of the two groups have been immunized four times with an interval of two weeks between two immunizations.

The blood was collected from the orbit of mice one week after the last immunization. The collection of the small intestine lavage fluid samples was described by Tochikubo et al. (1998). The serum and lavage fluid samples were stored at -20°C until use.

The detection of the titer of M2e antibody in the serum and the intestinal secretion

ELISA reaction was employed to detect the specific IgG against M2e-peptide in the serum and the specific IgA against M2e-peptide in the small intestine lavage fluid. To determine M2eHBc+ antibody titers, plates were coated with 100 μ l of a 2 μ g/ml M2e-peptide solution in 50 mM sodium bicarbonate buffer, pH 9.7, and incubated overnight at 4°C . After washing, the plates were blocked for 1 h with 200 μ l of 5% BSA in PBST. After 1 h incubation, the plates were washed again. A series of 1/2 dilutions of the different serum samples were loaded on the M2e-peptide-coated plates. The bound antibodies were detected with a horseradish peroxidase labeled antibody against mouse IgG (Huamei biology, diluted 1 : 4000 in PBST). The detailed operation process referred to aforementioned GM1-ELISA. Endpoint titers are defined as the highest dilution producing an optical density value twice that of background (negative serum). A similar procedure was followed for the assay of specific M2eHBc+IgA in small intestine lavage fluid, except that the bound antibodies were detected with a peroxidase-labeled antibody directed against mouse IgA (eBioscience, diluted 1 : 4000 in PBST), and the optical density values at 450 nm were recorded to reflect

the level of specific mucosal IgA against antigen in the small intestine of immunized mice. Comparisons between experimental groups were made by Student's t-test and probability (p) values <0.05 were considered significant.

Results

The construction and expression of pM-M2eHBc+ and pM-LBM2eHBc+ prokaryotic expression vectors

M2eHBc+ fusion gene fragment was produced through over-lap polymerase chain reaction (Fig. 1). The amplified fragment was digested and ligated into the prokaryotic expression vector pMAL-c2x. The constructed vectors were verified by sequencing and named as pM-M2eHBc+ and pM-LBM2eHBc+, respectively.

pM-M2eHBc+ and pM-LBM2eHBc+ vectors were transformed into the Rosetta (DE3). The positive clones were incubated in the Luria-Bertani liquid medium with double antibiotics. After induction with 0.8 mg/l IPTG, two new protein bands of 66.5 kDa and 83.2 kDa (as indicated by the arrow) were observed in SDS-PAGE (Fig. 2). Their molecular weight were in accordance with that of the anticipated fusion proteins, suggesting that two fusion genes of M2eHBc+ and LBM2eHBc+ were expressed in *Escherichia coli* cell. The induction bacterial cells were lysed by sonication. The SDS-PAGE image showed that most of the target proteins existed in the supernatant indicating both proteins is in soluble form.

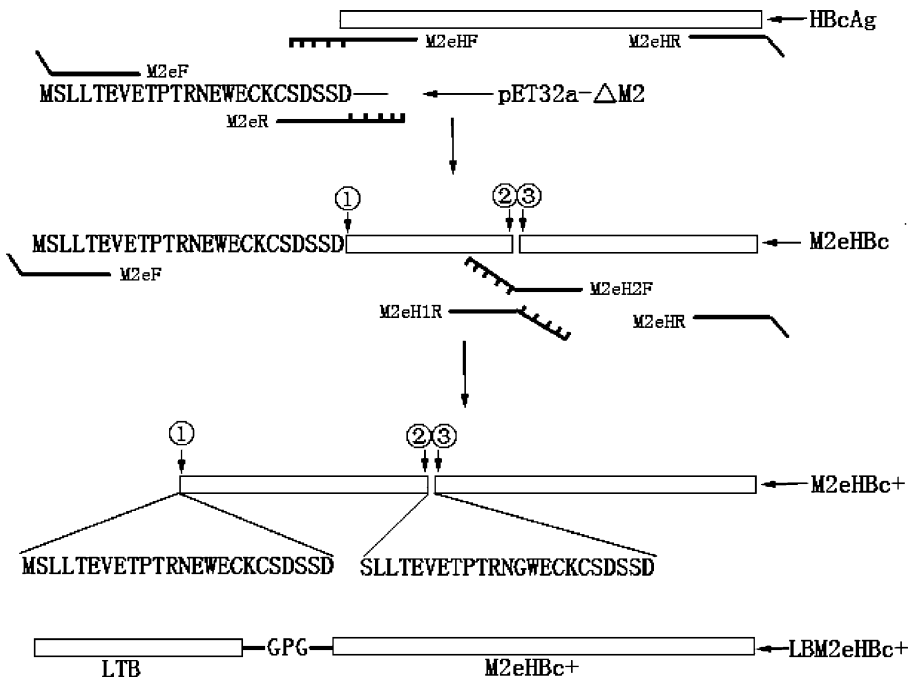


Fig. 1 The cloning strategy for constructing the fused gene M2eHBc+ and LBM2eHBc+. ①, ② and ③ indicate No. 2, No. 80 and No. 81 amino acid site of full HBcAg, respectively

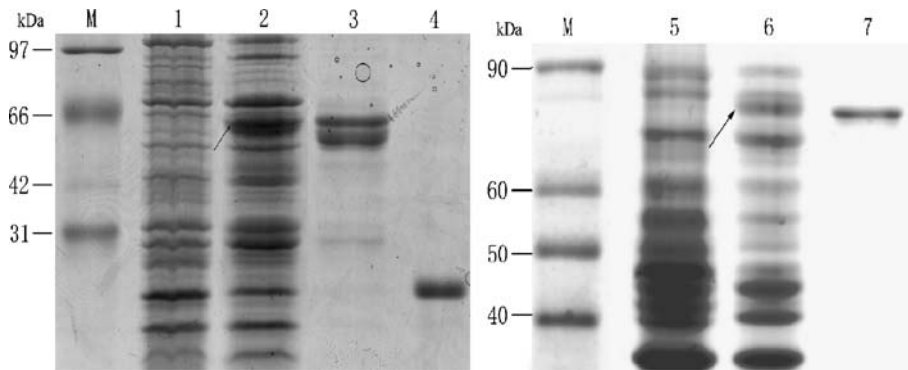


Fig. 2 The analysis of SDS-PAGE of expression of M2eHBc+ and LBM2eHBc+ fusion protein. The *Escherichia coli* Rosetta (DE3) strain hosting plasmid pM-M2eHBc+ and pM-LBM2eHBc+ induced with or without IPTG. Lane 1 and lane 2 were pM-M2eHBc+ induced without and with IPTG respectively; Lane 3 was primarily purified M2eHBc+ using an amylose resin; Lane 4 was M2eHBc+ without maltose tag; Lane 5 and lane 6 were pM-LBM2eHBc+ induced without and with IPTG respectively; Lane 7 was primarily purified LBM2eHBc+ using an amylose resin; M was low molecular weight protein marker

After proteins purification and the removal of maltose-binding protein tag, the target proteins with the purity higher than 95% was obtained.

The antigenicity analysis and GM1-ELISA for the recombinant protein

As shown in the Western blotting of M2eHBc+ and LBM2eHBc+ (Fig. 3), both M2eHBc+ and LBM2eHBc+ can be recognized by H5N1 type M2 antibody, indicated by the specific hybridization signals on the nylon membrane, while no signal was observed in the negative control lane. Therefore, both fusion proteins inherited the antigenicity of M2e epitope.

Also the results from GM1-ELISA experiment (Fig. 4) show that only the LBM2eHBc+ protein and GM1 combination produced high absorption signal at 450 nm that comparable to the positive control, the GM1/CTB combination. Both two negative controls, to the

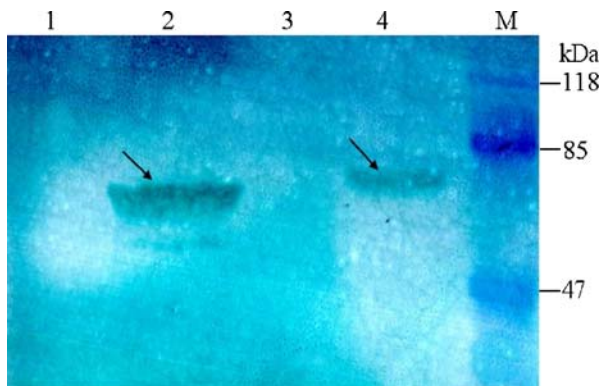


Fig. 3 Western blotting analysis of M2eHBc+ and LBM2eHBc+ fusion protein with rabbit anti-H5N1-M2 antibody. Lane 1 and lane 2 were the total protein of *Escherichia coli* Rosetta (DE3) strain hosting plasmid pM-M2eHBc+ induced without and with IPTG for 4 h respectively; Lane 3 and lane 4 were the total protein of *Escherichia coli* Rosetta (DE3) strain hosting plasmid pM-LBM2eHBc+ induced without and with IPTG for 4 h respectively; M was prestained protein molecular weight marker

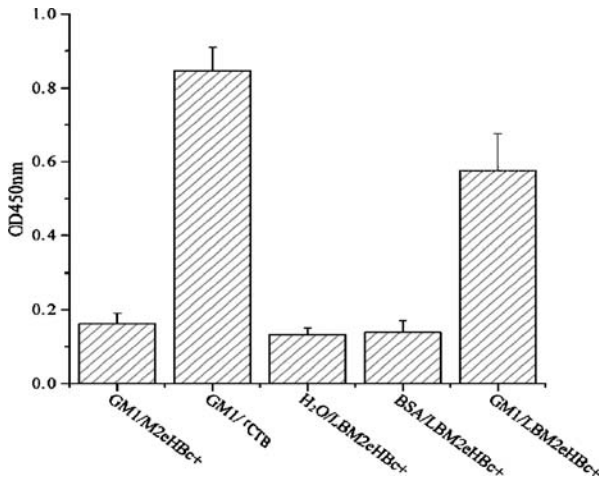


Fig. 4 GM1 binding assay of recombinant LBM2eHBc+ protein. The ELISA was performed by coating the plates with either ganglioside receptor GM1, or BSA and H₂O as receptor molecules. Binding activity is expressed as the arithmetic means of three duplicates optical density values and standard deviations (vertical lines)

combination of LBM2eHBc+/H₂O and LBM2eHBc+/BSA show low background absorption. The M2eHBc+/GM1 combination produce an absorption equivalent to the negative controls. The student's t-test shows the difference between the GM1/LBM2eHBc+ combination and the negative controls is significant. Thus, only the recombinant LBM2eHBc+ protein can binding to GM1.

Transmission electron microscope observation

Under the samples negative staining and the transmission electron microscope, it was observed that the purified LBM2eHBc+ and M2eHBc+ protein can both self-assemble into VLP and the diameter was about 25 nm (Fig. 5).

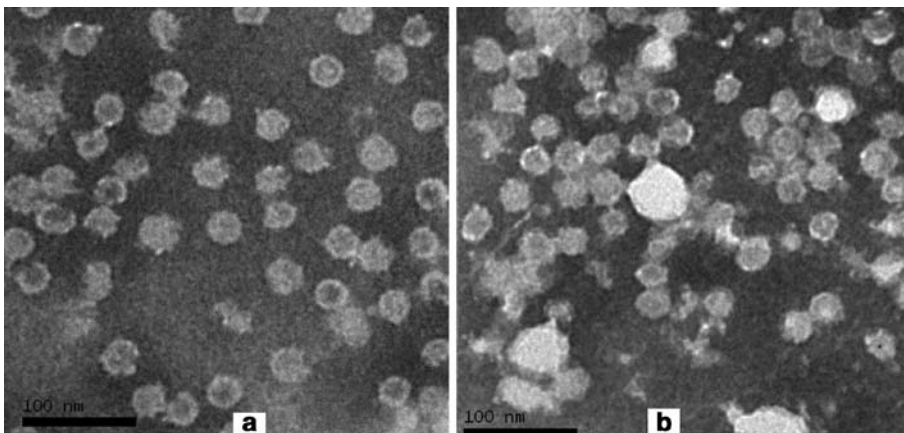


Fig. 5 Negative staining transmission electron microscopy of VLP formed from proteins being expressed by the constructs (a) pM-M2eHBc+ and (b) pM-LBM2eHBc+. Scale bar, 100 nm

Enhancement of mucosal immunogenicity of M2eHBc+ by fusion LTB co-expression

At one week after final immunization, antisera and small intestine lavage fluid samples were collected and subjected to ELISA against recombinant M2eHBc+ protein to determine specific IgG and IgA antibody titers (Figs. 6 and 7). Results showed that immunization with M2eHBc+ (i.n.) alone can induce the moderate antigen-specific IgG and sIgA in the mice. The difference was significant in comparison with the negative control (immunization with PBS) ($p < 0.05$). The immune efficacy in the LBM2eHBc+ immunization group (i.n.) was the highest. The co-expression of LTB markedly enhanced the immunogenicity of M2eHBc+ in comparison with the immunization with M2eHBc+ (i.n.) alone ($p < 0.05$), producing an enhanced increase in anti-M2eHBc+ IgG and sIgA titers in serum and small intestine lavage of LBM2eHBc+ immunized mice.

The producing tendency and significance of the antibody in different treatment groups in the oral administration route were almost similar to those of the intranasal dropping group, but the titer of the M2e-peptide antibody showed a certain extent of decrease. M2eHBc+ alone (o) can induce the production of the antigen-specific IgG in the mice, but it can not effectively induce the production of antigen-specific sIgA in the mucosal tissue. In contrast, LBM2eHBc+, the co-expression product of LTB and M2eHBc+, can not only significantly enhance the production of anti-M2e-peptide serum IgG in the mice, but also significantly enhance the secretion of anti-M2e-peptide mucosal sIgA in the mice by oral immunization. The difference was significant when compared to the M2eHBc+ alone oral immunization ($p < 0.05$). Above results of the immunization in the mice showed that LTB can both significantly enhance the production of serum IgG and mucosal sIgA to the co-expressed antigen M2eHBc+ by the both mucosal immunization routes.

Discussion

VLP consists of viral proteins derived from the structural proteins of a virus. These proteins can self-assemble into virus capsids without infectious competence in many expression

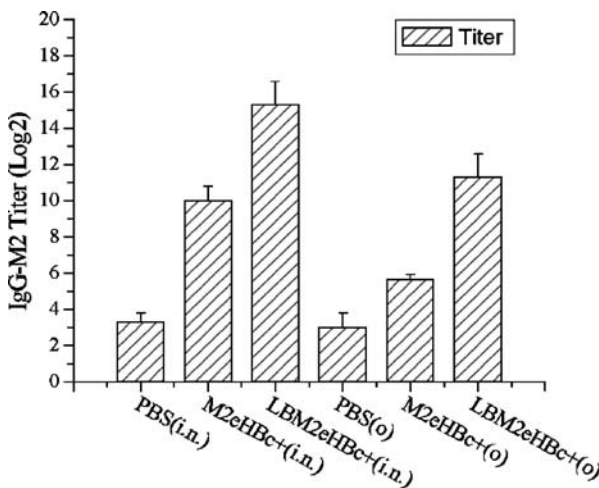


Fig. 6 The titer of IgG-M2e in the different antigen immunized mice sera. Antibody titers are expressed as the geometric mean titers and standard deviations (vertical lines)

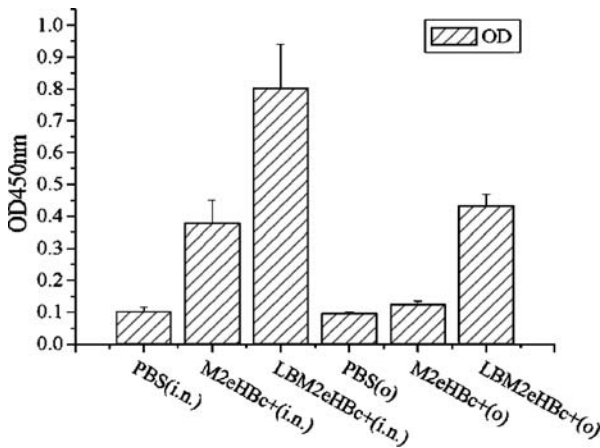


Fig. 7 The titer of IgA-M2 in the different antigen immunized mice intestine lavage fluids. Antibody titers are expressed as the arithmetic means of immunized mice intestine lavage fluids optical density values at 450 nm and standard deviations (vertical lines)

systems. VLP used as vaccines was often effective at eliciting both T cell and B cell immune responses. Therefore, VLP could serve as molecular carrier to effectively display immunodominant epitopes on the outer surface of the particle (Ulrich et al. 1998). HBcAg is an essential structural protein of hepatitis B virus, and also a widely used versatile carrier for heterologous epitopes (Ulrich et al. 1998). HBcAg particles not only possess an outstanding ability to induce B cell, T helper and cytotoxic T cell response, but also are remarkably tolerant to modifications such as substitutions, insertions, and deletions in its N terminal, MIR and C terminal region. The HBcAg particles permit the inserted polypeptides to display the appropriate immunological properties, while still holding the efficient particle formation and high-level yields in practically all popular homologous and heterologous expression systems (Ulrich et al. 1998; Pumpens and Grens 2001). Several investigations have showed that M2e became highly immunogenic when fused to N terminal or MIR of hepatitis virus B core. Immunization with these fusion proteins through mucosal and other immunization routes resulted in protection against the homologous or heterologous lethal influenza virus challenge (Neirynek et al. 1999; De Filette et al. 2005, 2006). Moreover, this protection is ascribed to antibodies as it is transferable by passive immunization (Neirynek et al. 1999). As described in this study, pM-M2eHBc+ expression vector was made by inserting the M2e sequence simultaneously in the N terminal and MIR region of HBcAg, and then LTB was further fused at the N terminal of M2eHBc+ to construct the pM-LBM2eHBc+. Two fusion genes can both efficiently expressed in the prokaryotic expression system, and the expression products can self-assemble into VLP, meaning that M2e epitope was displayed on the outer surface of VLP as desired. The simultaneous insertion of M2e into the two sites of HBcAg and the subsequent fusion of LTB in the N terminal of M2eHBc+ did not impair the self-assembly competence of HBcAg particle. In one previous report, fusion of a 120 aa peptide originated from the hantavirus nucleocapsid to the N-terminus of HBcAg prevented self-assembly (Koletzki et al. 1999). The capacity of N-terminal HBcAg for the insertion peptide has been estimated as around 50 aa (Pumpens and Grens 2001). However, we have shown in this study that a total of 148 aa has been inserted into the N terminal of HBcAg protein and the resulted fusion protein is yet able to self-assemble into particles as shown in the transmission electron microscope photos. The

difference might be due to a full-length HBcAg we used in this study, while the earlier employed a truncated HBcAg with a deletion of No. 150–183 aa protamin-like arginine-rich domain in the C-terminal of HBcAg protein. Truncated HBcAg particle and entire HBcAg particle were practically indistinguishable as shown by electron cryomicroscopy. However, the latter particles possess stronger self-assembly potency and better stability (Crowther et al. 1994). Furthermore, the linker polypeptide (GPG) at the junction between LTb subunit and M2eHBc+ may function as a flexible linker that allows the two connected peptides to fold correctly. Using a flexible linker is also considered as a strategy for overcoming the limitation of the amino acid insertion fragment length in the N terminal of HBcAg (Karpenko et al. 1997).

There is widespread interest in i.n. vaccination in AIV vaccine development. Vaccines can be safely and rapidly administered to a large number of recipients through the i.n. route, which may be more efficient in stimulating mucosal lymphoid tissues and leads to improved mucosal immunity in the respiratory tract region. In general, the AIV in animals germinated from the upper respiratory tract by air spread. Vaccines, most of which aimed at inducing serum antibody responses and are generally administered intramuscularly, can not provide adequate protection in the upper respiratory tract against AIV infection (De Haan et al. 2001). Therefore, new-generation influenza vaccines should aim at induction of a mucosal antibody response in the upper respiratory tract and other mucosal tissue in addition to systemic IgG. LTb has been used extensively as a mucosal immunoadjuvant for intranasal and oral vaccination. In some investigations LTb manifested potent mucosal immunoadjuvant activity not only when administered to mice in conjunction with influenza virus subunit vaccine (Verweij et al. 1998; De Haan et al. 2001) but also administered to animals (mice, Leghorn laying hens and carp) with purified products co-expressed in *Escherichia coli* (Yamanaka et al. 2006), yeast (Fingerut et al. 2005) or plant (Companjen et al. 2006), respectively. De Filette et al. (2005, 2006) successively reported that two types of mucosal adjuvants, non-toxic variants of LT (R192G) and CTA1-DD, had potent mucosal adjuvant activity to particle shape vaccines of M2e-HBc. In our study, when i.n. administered, LBM2eHBc+ elicited strong antigen-specific serum antibody and mucosal sIgA responses. The response is considerably higher than that induced by M2eHBc+ antigen alone, which is strong evidence that LTb can be used as an enhancement factor for M2eHBc+ induced mucosal immune system responses (Fig. 7). This result is also consistent with aforementioned reports (Verweij et al. 1998; De Haan et al. 2001; Yamanaka et al. 2006; Fingerut et al. 2006). As mucosal adjuvant LTb and antigen M2eHBc+ have been fused in one expression plasmid, tedious job in vaccine preparation process can be alleviated, compared to the methods described by De Filette et al. (2005, 2006).

Oral vaccine has several advantages, such as safer, more convenient, as well as easier supplement to food in animal immunization. However, the content-rich proteinase and lowest pH environment in gastrointestinal tract during oral administration is not suitable for the stability of immune protein, limiting the efficacy of oral vaccine. The currently reported oral vaccines, except for live vaccines, all have to use mucosal adjuvants, such as LT or CT derivatives (Lauterslager and Hilgers 2002). In this study, M2eHBc alone can stimulate mice to produce moderate M2e-peptide antibodies IgG, but not sIgA through oral route. In LBM2eHBc fusion protein immunization group, the result indicated that LTb can significantly increase M2eHBc induced serum and mucosal immune response through oral vaccination. We also noticed that when the dose of oral vaccine is twice as much as intranasal immunization, the titer of antibody produced in each protein immunization group is lower than that of intranasal immunization group. The main reason for that oral vaccine

requires large dose of antigen is likely that the above mentioned gastrointestinal environment partially degraded the immune protein. Further optimization is needed for the oral administered immunization.

In summary, we produced LBM2eHBc+ fusion proteins which can self-assemble into VLP structure with GM1 binding activity in *in vitro* experiments. The immunization of mice through two mucosal routes can effectively stimulate the mucosal immune responses and induce mice immune system to produce M2e-peptide specific IgG and mucosal sIgA. Previous investigations have shown that LTB and CTB were effective mucosal adjuvants in the immunization tests in chicken (Fingerut et al. 2005, 2006). Although hasn't been tested, we reasonably expect that LBM2eHBc+ protein can also effectively induce the immune responses of chicken either by intranasal dropping or by eye dropping immunization routes, which will be studied in the following projects.

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