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Reverse micelle-encapsulated recombinant baculovirus as an oral vaccine against H5N1 infection in mice

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ABSTRACT

Induction of mucosal immunity through oral immunization is an effective way to control influenza infection. In this study, baculovirus displaying influenza hemagglutinin was encapsulated within a reverse micelle structure of phosphatidylcholine and delivered into the gastrointestinal tract of mice to study its efficacy as an oral vaccine against cross-clade H5N1 infection. Mice vaccinated with encapsulated baculovirus displaying HA (En-BacHA) showed significantly enhanced HA specific serum IgG and mucosal IgA antibodies, and higher hemagglutination inhibition (HI) titers, when compared to its non-encapsulated form (BacHA). Estimation of serum neutralizing antibodies also indicated that En-BacHA formulation was able to induce strong cross-clade neutralization against heterologous H5N1 strains (clade 1.0, clade 2.1, clade 4.0 and clade 8.0). Further, mice vaccinated with En-BacHA alone were able to confer 100% protection against 5 MLD50 of HPAI heterologous H5N1 strain (clade 1). Inclusion of recombinant cholera toxin B subunit as a mucosal adjuvant in the vaccine formulation did not show any significant effect in both systemic and mucosal immune responses. Oral delivery of encapsulated recombinant H5 HA expressed on baculovirus surface is an effective way to prime the immune system against H5N1 infection in mice and will have no biosafety concerns associated with their production or administration.

1. Introduction

Influenza is a highly contagious viral respiratory disease that causes significant morbidity and mortality worldwide each year (de Jong and Hien, 2006). Current pandemic situation with new H1N1 swine-origin influenza A virus (S-OIV) has stressed the urgent need for safe and effective vaccines (CDCP, 2009). At present, influenza vaccines are generally administered via subdermal or intramuscular route to stimulate systemic immune response to prevent the disease (Ruat et al., 2008; Gao et al., 2006). However, for infection acquired through respiratory system, mucosal immune response plays an important role in protecting the host at the port of entry (Cox et al., 2004). Mucosal immunization, which is shown to stimulate both mucosal and systemic immune responses, would be an effective way to control the infection by influenza viruses (Ogra et al., 2001). Despite the recent attention towards intranasal administration to enhance mucosal immune responses, oral vaccination is still considered as the most effective way to increase patient compliance (Mann et al., 2004; CDCP, 2004). Non-invasive, pain-free self-administration, affordability, improved logistics and mass coverage during pre-pandemic and pandemic situations make oral vaccination an attractive option.

Several studies have previously reported the use of inactivated whole-virus, split or subunit antigens to study the efficacy of oral immunization against influenza (Amorij et al., 2007; Bender et al., 1996). Though some of these vaccines have proven to be effective, the production of these vaccines still possess some safety and technical issues as most method still relies on the culture of live viruses in embryonated chicken eggs. Moreover, the current method of producing influenza vaccines is technically challenging owing to the constraints in using high level biocontainment facilities and thorough inactivation procedures. Moreover, inactivated whole-virus and split-virus vaccines were shown to activate CD81 cytotoxic T-lymphocyte responses only rarely, have poor cross-reactivity to antigenic variants, and produce poor secretory immunoglobulin A (IgA) responses (Barackman et al., 2001).

Baculovirus expression system has long been used to produce recombinant proteins due to the proper post-translational modifications and high yield in insect cells (He et al., 2009). HA protein

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produced in the baculovirus expression system has been extensively evaluated in humans as influenza vaccines (Treanor et al., 2007). However, influenza HA expressed in insects cells is highly hydrophobic and its low solubility increases the difficulty of purification reducing its effectiveness as vaccines (Treanor et al., 2001). Baculovirus surface display technology has recently evolved as a novel platform for vaccine development against influenza viruses. This system enables the presentation of large complex proteins on the surface of baculovirus particles in their native functional conformation (Peralta et al., 2007), resulting in superior immune response when used as immunogens. More importantly, baculovirus is naturally replication-incompetent in mammalian cells (Tani et al., 2003) and even do not require the use of any live influenza viruses during vaccine development, manufacturing and administration processes. As an alternative to conventional egg-based influenza vaccines, the baculovirus expression system exploits serum-free insect cell suspension culture resulting in simplified cultivation and purification procedures. Combining the advantages of both baculovirus surface display technology and oral immunization for the development of influenza vaccines will have the added advantage of safety and immunogenecity.

The only major obstacle associated with the oral immunization approach is that the antigen in the vaccine formulation can be substantially degraded by gastric hydrochloric acid and proteolytic enzymes present in the gut (Pauletti et al., 1996), resulting in poor immunogenicity. Hence, in the present report, we have described a novel approach to deliver baculovirus displaying HA into the gastrointestinal tract of the mice using a reverse micelle-based carrier vehicle. This strategy will entrap the antigen within a particulate structure and prevents them from being exposed to the destructive gut components. We have also assessed the effect of BacHA/vaccine oil formulation with rCTB as mucosal adjuvant.

2. Materials and methods

2.1. Influenza viruses

The highly pathogenic influenza A Human H5N1 viruses such as CDC/669/Indonesia/06 (GeneBank accession no. CY014481), CDC/594/Indonesia/06 (GeneBank accession no. CY014272) were obtained from Ministry of Health (MOH), Indonesia. The H5N1 viruses from different phylogenetic clades were rescued by Reverse Genetics (WHO, 2005). Briefly, the hemagglutinin (HA) and neuraminidase (NA) genes of H5N1 viruses from clade 1.0 (A/Vietnam/1203/2004), clade 2.1 (A/Indonesia/CDC1031/2007, clade 4.0 A/goose/Guiyang/337/06 and clade 8.0 (A/chicken/Henan/12/2004) were synthesized (GenScript) based on the sequences from the NCBI influenza Database. The synthesized HA and NA genes were cloned into a dual-promoter plasmid for influenza A reverse genetics (Prabakaran et al., 2009). The reassortant viruses were rescued by transfecting plasmids containing HA and NA together with the remaining six gene plasmids derived from A/Puerto Rico/8/34 (H1N1) into co-culture of 293T and MDCK cells using Lipofectamine 2000 (Invitrogen Corp.). The HA and NA genes of reassortant viruses were sequenced to confirm the presence of introduced HA and NA genes. Stock viruses were propagated in the allantoic cavity of 11-day-old embryonated eggs and the allantoic fluid was harvested and stored in aliquots at −80 °C. Virus content was determined by standard hemagglutination (HA) assay. Recombinant cholera toxin B subunit (rCTB) was provided by Shanghai United Cell Biotechnology Co. Ltd. (Shanghai, PR China).

All experiments with high pathogenic viruses were conducted in an animal biosafety level 3 (ABSL-3) containment facility, in compliance with CDC/NIH and WHO recommendations (NIH, 1999).

2.2. Generation of recombinant baculovirus vaccine

For the construction of recombinant baculovirus BacHA, the full length ORF of HA gene was amplified from two different influenza H5N1 Indonesia strains (CDC/669/Indonesia/06 and CDC/594/Indonesia/06). The original pFASTBacHT A (Invitrogen, San Diego, CA) baculovirus transfer vector was modified by deleting the original polyhedrin promoter sequence including Histidine tag with AccI and RsrII restriction enzymes. The ie1 promoter was amplified from White Spot Syndrome Virus (WSSV) DNA and then inserted into pFASTBacHT A using AccI and RsrII restriction sites. The HA gene was then inserted into the transfer vector using RsrII and Hind III restriction sites. For the generation of recombinant baculoviruses the constructs were integrated into the baculovirus genome within DH10Bac^{TM} (Invitrogen) through site-specific transposition using Bac-To-Bac system (Invitrogen). Recombinant baculovirus were then propagated in SF-900II SFM (Gibco BRL) at 27 °C by infecting 200 ml of Sf9-cells in suspension at a cell density of 2×10^6 cells/ml with a multiplicity of infection (MOI) of 0.5. The budded virus particles released into the media were harvested at 4 dpi and filtered through 0.22 μm filter for infection experiments. The viral titers were determined by plaque assay and the virus stock was stored at 4 °C. A large-scale amplification of BacHA was carried out and the virus particles were purified by two rounds of sucrose gradient following the standard protocols (O'Reilly et al., 1992). Recombinant baculovirus was inactivated with binary ethylenimine (BEI) as described previously (Rueda et al., 2000). The complete loss of infectivity of the inactivated BacHA was determined by infection of Sf9 cell monolayer and observation of cytopathic effects for at least 7 days. For determining the HA content in recombinant baculovirus, single radial immuno-diffusion (SRID) assay was carried out according to Wood et al. (1977) using antiserum for influenza H5N1 and purified HA protein (Protein Sciences Corporation) as a standard.

2.3. Immunoblot analysis

Budded Baculovirus particles displaying HA were fractionated on SDS-PAGE and the separated proteins were transferred onto nitrocellulose membrane and blocked with 5% non-fat milk in PBST (1 × PBS and 0.1% Tween-20) for 1 h at room temperature. The membrane was incubated with guinea pig anti-HA polyclonal antibodies at a dilution of 1:500, rinsed and incubated subsequently with HRP-conjugated rabbit anti-guinea pig (DakoCytomation, Denmark) for 1 h at room temperature. The membrane was washed and developed by incubation with 3,3′-diaminobenzidine (DAB) and hydrogen peroxide, as described previously (Gallagher et al., 2004).

2.4. Confocal microscopy

The Sf-9 cells were cultured on sterile cover slips in 6-well plates and infected at an MOI of 0.5. Forty-eight hours post-infection, cells were fixed with 4% paraformaldehyde (w/v) for 15 min at room temperature, rinsed with PBS, and blocked with 1% gelatine for 30 min. The cells were then incubated with the guinea pig anti-HA polyclonal antibody (1:100 dilution) for 1 h at room temperature, followed by incubation with FITC-conjugated rabbit anti-guinea pig (DakoCytomation, Denmark) at a dilution of 1:100. Protein localization was visualized using a confocal microscope (Carl Zeiss LSM 510, Germany).

2.5. Preparation of vesicle formulation

For formulation, soya phosphatidyl choline (supplier Lucas Meyer) was dispersed to form liposomes in distilled water at

a concentration of 100 mg/ml, and extruded through a 0.2 μm straight-pore Anotop membrane filter. To 3 ml of liposome suspension, 100 μl of recombinant baculovirus displaying HA (2.5 mg HA/ml) were added, mixed well, frozen, and then lyophilized overnight at a vacuum of 1 mbar or less. The following day, 500 μl of mineral oil (Huls AG, Germany) was added to the dry residue, and mixed gently at room temperature until fully dissolved to get a final concentration of 250 μg HA/500 μl mineral oil. Thus a single dose of 25 μg HA antigen (optimal concentration determined from previous trials) was contained in 0.05 ml mineral oil. In addition, for adjuvanted group, rCTB was added to the baculovirus stock and the formulation was prepared as described above. Controls containing just antigen or rCTB alone were prepared by omitting one or other of the appropriate components above. The product was stored at −4 °C under nitrogen until ready for use.

2.6. Oral immunization

Specific pathogen-free female BALB/c mice (6-week old) were obtained from the Laboratory Animals Centre, National University of Singapore, and maintained at the Animal Holding Unit of the Temasek Life Sciences Laboratory, Singapore. Prior to immunization all mice were starved for 2 h, otherwise food and water were supplied *ad libitum*. Ten mice per each experimental group ($c = 10$/group) received intragastric gavage on days 0, 7 and 14 with 50 μl of encapsulated baculovirus displaying hemagglutinin (En-BacHA) or non-encapsulated form (BacHA) containing 25 μg of HA (approximately 10^8 plaque forming unit of baculovirus particles/dose), adjuvanted with or without rCTB. Sera from each group ($n = 5$) of mice were collected on days 14 and 42, and 5 mice from the same group were sacrificed on day 28 to collect serum and mucosal wash. The HA specific antibody of systemic and mucosal compartments were assessed by indirect ELISA and hemagglutination inhibition assay. The efficacy of the vaccine was assessed by host challenge against clade 1.0 HPAI H5N1 influenza strain. All animal experiments were carried out in accordance with the Guidelines for Animal Experiments prescribed by National Institute of Infectious Diseases (NIID) and experimental protocols were reviewed and approved by Institutional Animal Care and Use Committee at Temasek Life Sciences Laboratory, National University of Singapore.

2.7. Measurement of anti-H5 HA specific antibodies by indirect ELISA

The levels of mucosal IgA and serum IgG-specific antibodies against rHA0 antigen were determined separately by indirect enzyme-linked immunosorbent assay according to the method previously described (Bright et al., 2008). Briefly, 96-well ELISA plates were coated with purified, optimal concentration of 400 ng of recombinant H5HA per well in carbonate buffer. Samples of test serum were diluted serially two fold in 3% non-fat dry milk/PBS containing 0.05% Tween 20 (PBST) and mucosal wash were diluted directly 1:20. Then, the color development was visualized by adding goat anti-mouse IgG (Sigma) and goat anti-mouse IgA (Bethyl Lab) conjugated with horseradish peroxidise to the respective wells and followed by addition of 3,3′,5,5′-tetramethyl benzidine (Sigma). The reaction was stopped with 25 μl of 1 M sulphuric acid and the absorbance was measured at 450 nm using microwell plate reader. The mean absorbance value for triplicate wells was used to express serum antibody level.

2.8. Hemagglutination inhibition assay

The serum neutralization activity was measured by hemagglutination inhibition assays (Webster et al., 1991). Receptor destroying enzyme (RDE)-treated (Bright et al., 2006) sera were serially diluted (two fold) in V-bottom 96-well plates. Approximately 4 HA units of viral antigen was incubated with the serum for 30 min at room temperature, followed by the addition of 1% cRBCs and incubation at room temperature for 40 min. The inhibition of hemagglutination at the highest serum dilution was considered the HI titer of the serum.

2.9. Challenge test against influenza H5N1 virus infection

Four weeks after final vaccination, mice were transferred into animal BSL3 containment facility. Five mice per group were challenged intranasally with 5 MLD_{50} (mouse lethal dose 50%) of heterologous (Vietnam/1203/2004 clade 1.0 and) HPAI H5N1 strain. Fifty percent mouse lethal dose (MLD_{50}) of the influenza virus required for intranasal challenge experiments was predetermined. To determine the effect of oral delivery of en-BacHA with or without rCTB, mice were observed daily to monitor body weight and mortality. Monitoring continued until all animals died or until day 14 after challenge. All challenge experiments were conducted at animal biosafety level 3 containment facility.

2.10. Statistical analysis

The data are expressed as arithmetic mean ± standard error (SE). The unpaired two-tailed Student's *t*-test was performed to determine the level of significance in the difference between means of two groups. One-way ANOVA was also used to test for differences between groups, and Tukey HSD post hoc test was used to determine which groups were significantly different from the rest. All statistical analysis was done with SigmaStat 2.0® (Jandel Corporation) software. The level of significance was expressed as $P < 0.05$.

3. Results

3.1. Structural and antigenic conformation of baculovirus surface displayed HA0

Confocal microscopic analysis indicated that HA0 expressed by the recombinant baculovirus was able to successfully translocate to the plasma membrane of infected insect cells (Fig. 1A). Further western blot analysis and hemagglutination assay demonstrated that baculovirus surface displayed HA0 was able to sustain its authentic cleavage (Fig. 1B) and hemagglutinin activity (data not shown).

3.2. Systemic antibody response to the oral delivery of En-BacHA

Mice immunized orally with En-BacHA showed significantly enhanced HA specific serum IgG titers when compared to the BacHA vaccination. The presence of rCTB as adjuvant along with the En-BacHA or BacHA formulation did not show any significant improvement in antibody titers, when compared with the unadjuvanted En-BacHA or BacHA, respectively (Fig. 2).

Hemagglutination inhibition titers, which measures the efficacy of the antibody response to inhibit the functional ability of HA, was also obtained. The HI titers results showed that mice immunized orally with En-BacHA significantly enhanced the serum HI titer when compared to the BacHA on days 14, 28 and 42. However, serum from mice vaccinated with En-BacHA or BacHA combined with rCTB adjuvant did not have any significant difference on the HI titers over non-adjuvanted vaccine formulations (Fig. 3).

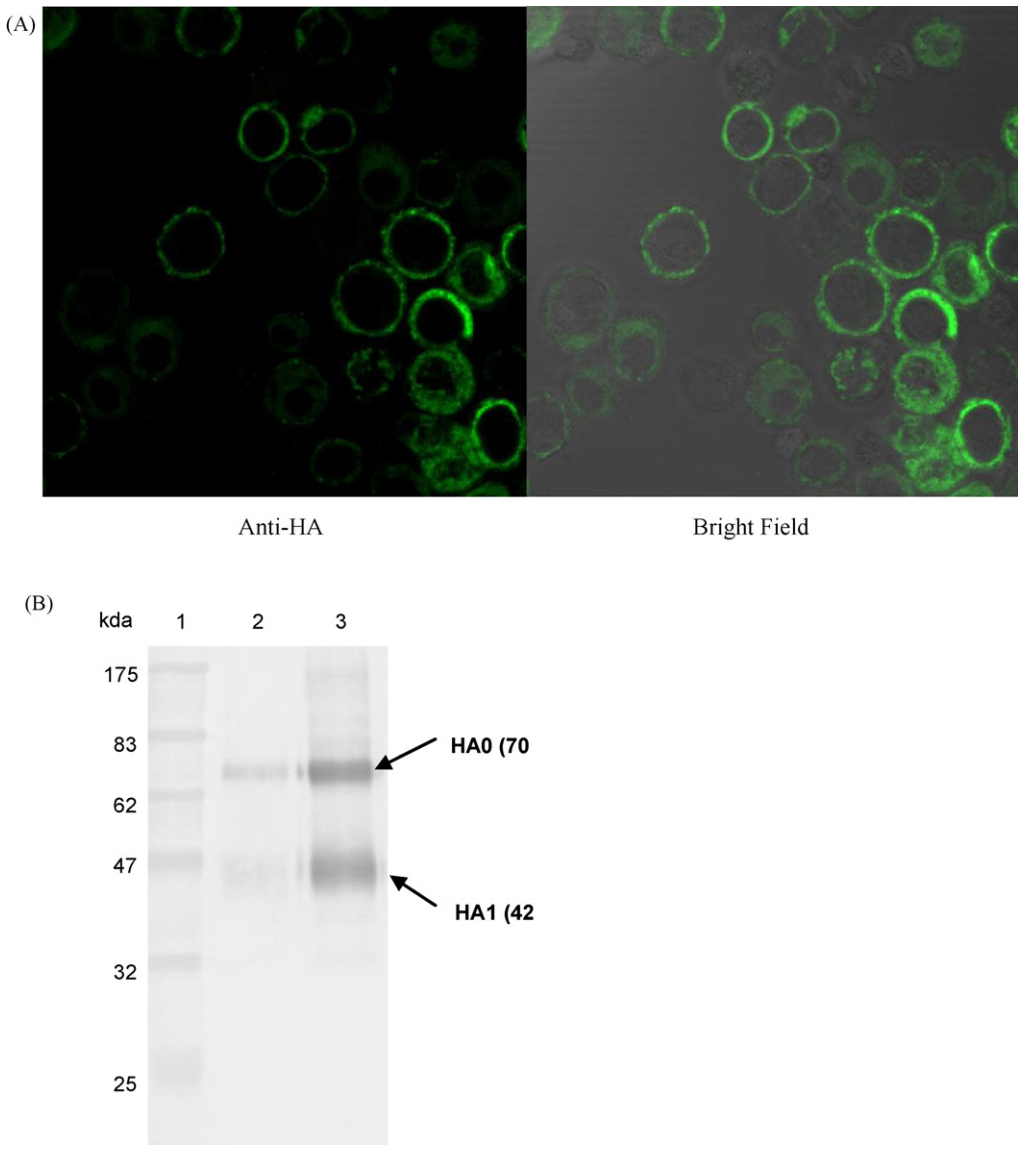

Fig. 1. Confocal microscopy and immunoblot analysis. (A) Confirmation of the expression of influenza hemagglutinin in Sf-9 cells by recombinant baculovirus. The cells were cultured on sterile cover slips and infected at a multiplicity of infection (MOI) of 0.5. Forty-eight hours post-infection, cells were fixed and stained with guinea pig anti-HA polyclonal antibody followed by FITC-conjugated secondary antibody. Images were captured by a Zeiss LSM 510 confocal microscope. (B) Immunoblot analysis of recombinant baculovirus showing the cleavage of HA0. Lane 1: pre-stained protein marker; lane 2: infected cell culture pellet; lane 3: infected cell culture supernatant containing budded baculovirus.

3.3. Mucosal immune response to the oral delivery

Indirect ELISA was performed to determine the HA-specific mucosal IgA levels on day 28 (4 weeks after the final immunization). Mice vaccinated with En-BacHA significantly ($P > 0.01$) enhanced the mucosal IgA levels compared to BacHA. Moreover, mice immunized with adjuvanted (rCTB) En-BacHA showed insignificant ($P < 0.05$) increase in mucosal IgA levels, when compared with the mice immunized with unadjuvanted En-BacHA. BacHA alone or in the presence of rCTB induced only very lower level of HA specific mucosal IgA antibody responses (Fig. 4).

3.4. Serum cross-clade neutralizing antibody titer to the oral delivery of En-BacHA

The serum neutralizing antibody titer against 100 CCID50 of different clades of H5N1 strains on day 42 showed that vaccination with En-BacHA alone significantly neutralized ($P < 0.01$) viruses from clade 1.0, clade 2.1, clade 4.0 and clade 8.0 compared with non-encapsulated BacHA (Fig. 5). The presence of the rCTB as adjuvant in both vaccine formulations did not significantly influence the neutralizing antibody titers when compared with unadjuvanted vaccination groups (Fig. 5).

3.5. Challenge studies after oral vaccination

Four weeks after final immunization, all groups of mice were challenged intranasally with 5 MLD_{50} of HPAI H5N1 strain from clade 1.0. Groups of mice immunized with En-BacHA with or without rCTB provided 100% protection against H5N1 virus challenge (Fig. 6). However, up to 8% loss of the original body weight was observed by day 3 after challenge (Fig. 7). Moreover, the group of mice that was co-administered with En-BacHA and rCTB regained their body weight more rapidly (within 5 days), compared to unadjuvanted En-BacHA. Mice vaccinated with BacHA alone showed about 22% loss of bodyweight on day 6 post-challenge and provided

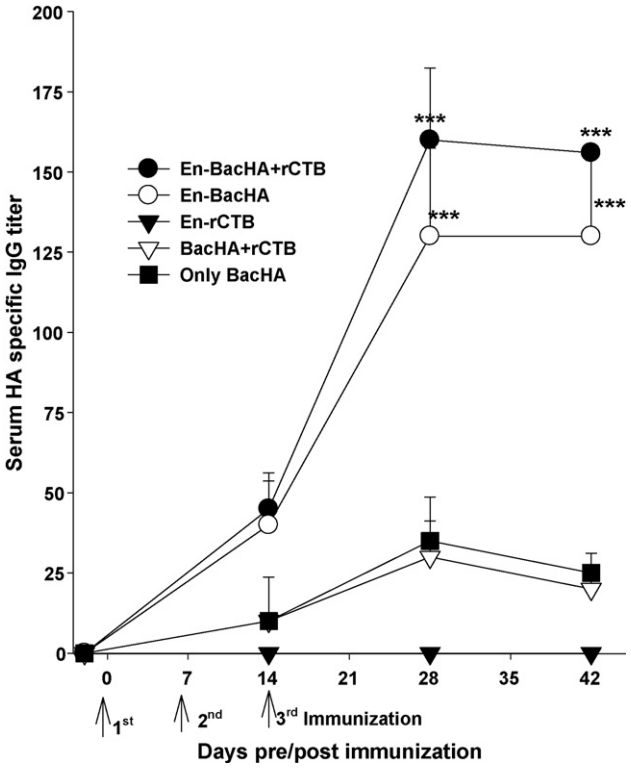

Fig. 2. Measurement of HA specific IgG antibody titers in the serum by indirect ELISA. Groups of mice ($n = 5$) were orally vaccinated three times on days 0, 7 and 14 with 50 μl containing 25 μg of En-BacHA or BacHA adjuvanted with or without rCTB. Each point represents the arithmetic mean value ($n = 5$) ± SD (***$P < 0.01$).

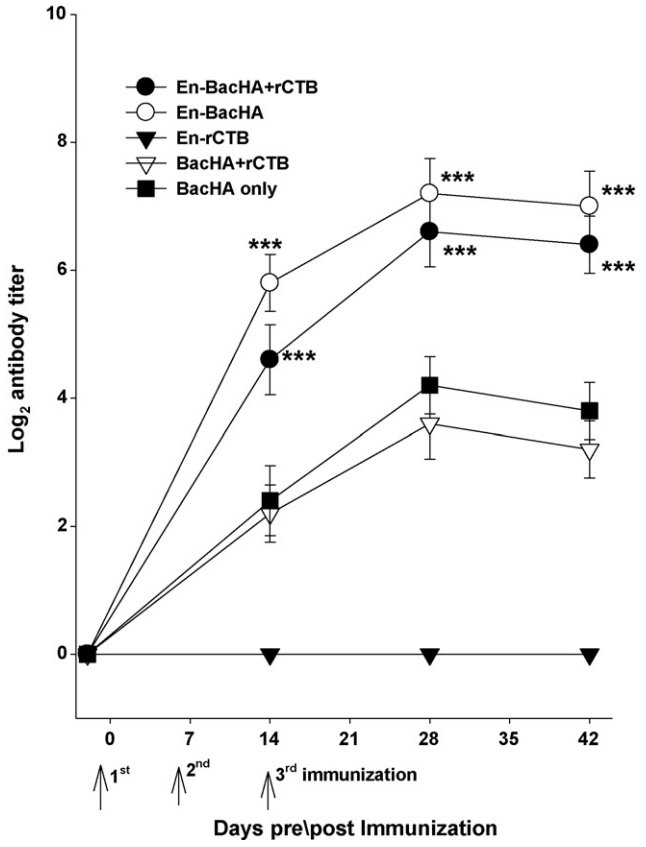

Fig. 3. Serum hemagglutination inhibition titer. Groups of mice ($n = 5$) were orally vaccinated three times on days 0, 7 and 14 with 50 μl containing 25 μg of En-BacHA or BacHA adjuvanted with or without rCTB. Each point represents the arithmetic mean value ($n = 5$) ± SD (***$P < 0.01$).

only 40% protection against 5 MLD_{50} of HPAI H5N1 virus. Similarly, mice co-administered with BacHA and rCTB showed at least 18% reduction in the total bodyweight and provided 60% protection against the virus challenge.

4. Discussion

Development of vaccination strategies that effectively induce mucosal immunity would be of major interest for preventing influenza infection. Previous studies have already reported the feasibility of oral vaccination to induce mucosal immune response (IgA) in the respiratory tract to confer protection against influenza viruses (Pang et al., 1992; Bender et al., 1996). Mucosal IgA responses have also been shown to exhibit cross-protective immunity against antigenically distinct viruses (Liew et al., 1984; Ito et al., 2003). In an effort to develop an oral vaccine against influenza infection, a recombinant baculovirus expressing influenza hemagglutinin (BacHA) was constructed with immediate early promoter derived from WSSV. Influenza hemagglutinin (HA), being a class II transmembrane protein, was naturally translocated to the plasma membrane of BacHA infected insect cells. Since WSSV ie1 promoter is independent of baculovirus encoded transcriptional and translational factors (Gao et al., 2007), HA is expressed immediately upon infection resulting in enhanced presentation on the baculovirus particles. Though insect cells generally trim the N-glycans of complex oligosaccharides found on the vertebrate glycoproteins (Kuroda et al., 1990), HA on the baculovirus surface has sustained its functional conformation as evidenced by its authentic hemagglutination activity and its cleavage into HA1 and HA2 subunits (Fig. 1A and B).

Baculovirus displaying HA is solubilized directly in the oil phase to create a structure similar to reverse micelle to provide protection against the destructive micro-environment in the intestinal lumen. In the encapsulated form, recombinant baculoviruses were entrapped within the polar head group of phosphatidylcholine, while the hydrophobic tail faces the external, continuous oil phase. This formulation is distinct from water-in-oil (W/O), oil-in-water (O/W) and multiple emulsions due to the absolute absence of water content in the preparation. Unlike conventional emulsions, this anhydrous encapsulation will prevent the antigen from denaturation within the formulation as well as during their gas-

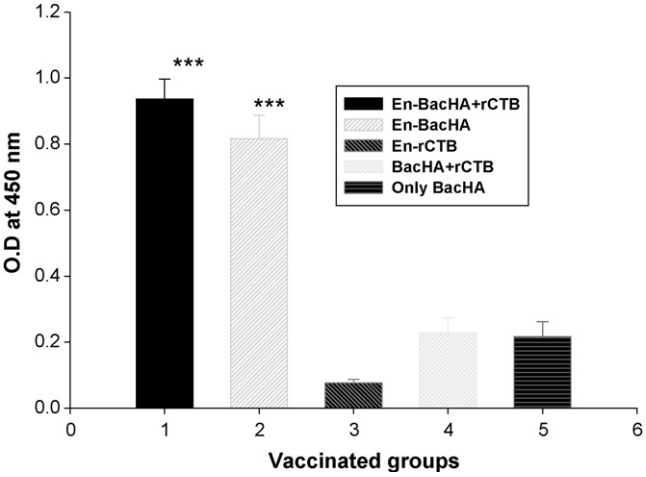

Fig. 4. Measurement of mucosal anti-HA specific IgA antibody levels by indirect ELISA. Groups of mice ($n = 5$) were orally vaccinated three times on days 0, 7 and 14 with 50 μl containing 25 μg of En-BacHA or BacHA adjuvanted with or without rCTB. Each point represents the arithmetic mean value ($n = 6$) ± SD (***$P < 0.01$).

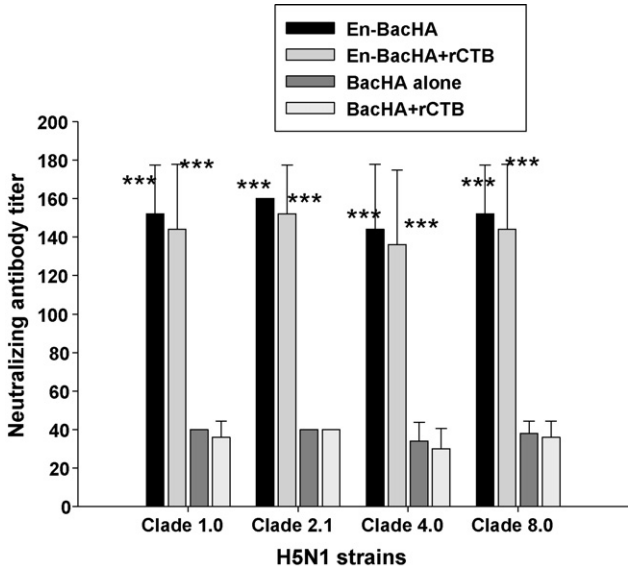

Fig. 5. Cross-clade serum microneutralization in mice. Groups of mice ($n = 5$) were orally vaccinated three times on days 0, 7 and 14 with 50 μl containing 25 μg of En-BacHA or BacHA adjuvanted with or without rCTB. The viruses from clade 1.0 (A/Vietnam/1203/2004), clade 2.1 (A/Indonesia/CDC1031/2007), clade 4.0 (clade 4.0 A/goose/Guiyang/337/06) and clade 8.0 (A/chicken/Henan/12/2004) were used for this study. The sera from the day of peak response, day 28 after the final immunization, were used for the assay. Each point represents the arithmetic mean value ($n = 6$) ± SE (***$P < 0.01$).

trointestinal transit upon oral vaccination (Domingos et al., 2008). Oral delivery of reverse micelle-encapsulated recombinant baculovirus (En-BacHA) containing 25 μg of HA was able to induce both systemic and mucosal immune responses in mice as indicated by high level of HA specific systemic (IgG) and mucosal (IgA)

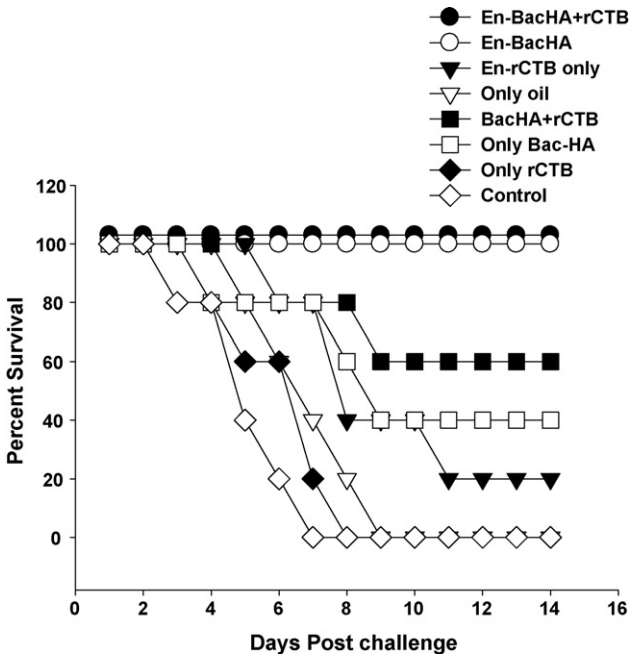

Fig. 6. Protection of mice from lethal H5N1 viral challenge. Groups of mice ($n = 5$) were orally vaccinated three times on days 0, 7 and 14 with 50 μl containing 25 μg of En-BacHA or BacHA adjuvanted with or without rCTB. Four weeks after the final vaccination, mice were intranasally infected with 5 MLD_{50} (mouse lethal dose 50%) of heterologous (Vietnam/1203/2004 clade 1.0) HPAI H5N1 strains. Mice were monitored for survival throughout a 14-day observation period. The results are expressed in terms of percent survival.

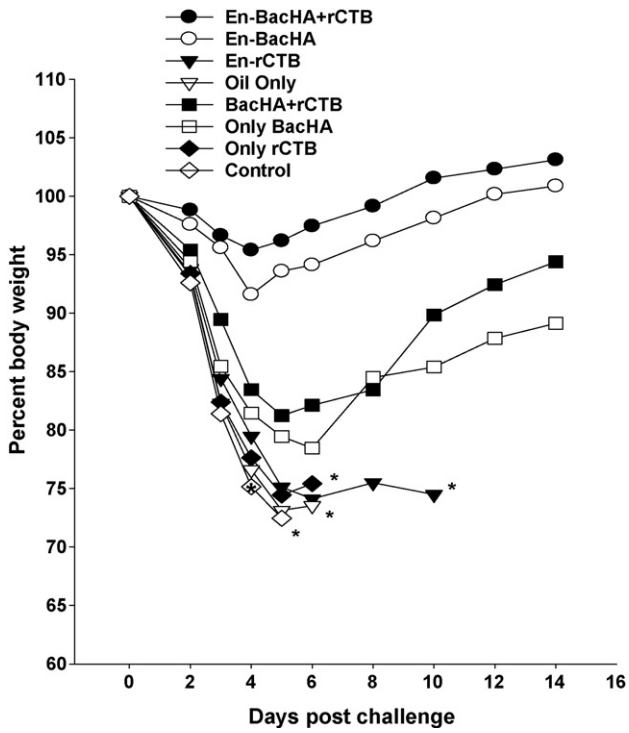

Fig. 7. Body weight of mice challenged with H5N1 virus. Groups of mice ($n = 5$) were orally vaccinated three times on days 0, 7 and 14 with 50 μl containing 25 μg of En-BacHA or BacHA adjuvanted with or without rCTB. Four weeks after the final vaccination, mice were intranasally infected with 5 MLD_{50} (mouse lethal dose 50%) of heterologous (Vietnam/1203/2004 clade 1.0) HPAI H5N1 strains. The group of mice challenged with Vietnam/1203/2004 clade 1.0 was also monitored for weight loss throughout a 14-day observation period (# represents no survival of any animals in the group). The results are expressed in terms of percent body weight (at the beginning of the trial).

antibodies respectively. However, non-encapsulated baculovirus (BacHA) at the same dosage was able to induce only low level of immune responses compared to its encapsulated counterpart. Standard influenza vaccines containing only 15 μg of HA were able to induce desired level of systemic antibody response when administered via the intramuscular route (Couch et al., 2007). However, induction of secretory IgA antibodies through intramuscular immunization was found to be limited. Further, Atmar et al. (2007) demonstrated that intranasal administration of inactivated trivalent vaccines in healthy young adults at a dose range of 15, 30 and 60 μg were able to enhance mucosal immune response in a dose-dependent manner. Nevertheless, the detrimental effects of intranasal vaccination to persons suffering from asthma, reactive airway disease and other chronic pulmonary or cardiovascular disorders (CDCP, 2004) makes oral vaccines the safest alternative (Mann et al., 2004).

The difference in the immune response conferred by the En-BacHA and BacHA is in accordance with the previous findings that oral delivery of soluble antigens were less immunogenic compared to the antigens encapsulated with oil emulsions (Conacher et al., 2001; Shahiwala and Amiji, 2008). Also protein antigens entrapped within the oil phase will result in its sustained release (Jansen et al., 2006), which could have favored strong humoral immunity. Further, exposed viral envelope proteins such as HA, which is naturally cleaved during the infection, is highly prone to proteolytic activity in the intestinal tract. This could have also accounted for the difference in the immune responses in mice vaccinated with the encapsulated and non-encapsulated forms of recombinant baculoviruses displaying HA.

En-BacHA was also able to induce high neutralization antibody titers in vivo in vaccinated mice, which efficiently neutralized 100 CCID50 of heterologus H5N1 strains from different clades 1.0, 4.0 and 8.0 compared with non-encapsulated baculovirus vaccine. This strong cross-clade immunity could be most likely due to the efficient delivery of encapsulated recombinant baculovirus into the intestinal mucosa, resulting in better antibody responses generated against intact conserved epitopes. Further, mice vaccinated with En-BacHA were completely protected against highly pathogenic heterologous H5N1 infection while the vaccination with BacHA alone provided only 40% survival rate. The body weight of mice vaccinated with En-BacHA, however, has been observed to reduce up to at least 8% when challenged with 5 MLD_{50} of H5N1 viruses. Interestingly, inclusion of rCTB in En-BacHA formulation facilitated the regain of body weight of the infected mice more rapidly compared to unadjuvanted En-BacHA. In our previous study with intranasal co-administration of BacHA and rCTB significantly enhanced both systemic and mucosal immune responses (Prabakaran et al., 2008). In contrast, oral delivery of En-BacHA combined with rCTB did not result in any significant effect in both mucosal IgA and serum IgG antibody responses. This is in accordance with previous findings, which reported that pure form of CTB does not always shows mucosal adjuvant activity but that it acts synergistically as a mucosal adjuvant together with a trace amount of whole cholera toxin (Takase et al., 1996; Tamura et al., 1994).

In summary, oral delivery of En-BacHA is efficacious in inducing both mucosal and systemic immune response and exhibits cross-protection against H5N1 viral infection. Reverse micelle mediated physical protection of the antigen along with delayed release features of the vaccine formulation could be responsible for improved antibody responses in mice vaccinated with En-BacHA. Further investigation is required to completely understand the mechanism by which En-BacHA can confer protection against influenza infection. One possible mechanism is the enhanced uptake of En-BacHA by Peyer's patches via specialized microfold (M) cells within the follicular associated epithelium (FAE) (Neutra et al., 2001) and processed by dendritic cells in the sub-epithelial dome (SED) for presentation to T cells, resulting in cell mediated immunity (Kelsall and Strober, 1996). The present study concludes that oral delivery of baculovirus displaying HA as a particualte structure will serve as an ideal choice for a vaccine in a pandemic and pre-pandemic situation in light of their safety and immunogenecity. This strategy does not require either high biocontainment facilities or tedious protein purification processes. In addition, phosphatidylcholine is a self-emulsifying amphiphile and hence, reduces the chances of local adverse reactions during the vaccination.

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