Gastrointestinal delivery of baculovirus displaying influenza hemagglutinin protects mice against heterologous H5N1 infection

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Abstract

The recent outbreaks of influenza A H5N1 in birds and humans have necessitated the development of potent H5N1 vaccines. In this study, we evaluated the protective potential of immediate early promoter-based baculovirus displaying HA (BacHA) against HPAI H5N1 infection in a mouse model. Gastrointestinal delivery of BacHA significantly enhanced systemic immune response in terms of HA specific serum IgG and hemagglutination inhibition titers. In addition, BacHA vaccine was able to significantly enhance mucosal IgA level. The inclusion of recombinant cholera toxin B subunit as a mucosal adjuvant along with BacHA vaccine did not influence both the systemic and mucosal immunity. Interestingly, inactivated form of BacHA was able to induce only negligible level of immune responses, when compared to its live counterpart. Microneutralization assay also indicated that live BacHA vaccine was able to induce strong cross-clade neutralization against heterologous H5N1 strains (clade 1.0, clade 2.1 and clade 8.0) compared to the inactivated BacHA. Viral challenge studies showed that live BacHA provided 100% protection against 5MLD50 of homologous (Clade 2.1) and heterologous (Clade 1) H5N1 infection. Moreover, histopathological examinations revealed that mice vaccinated with live BacHA had only minimal bronchitis in lungs and regained their body weight more rapidly post challenge. Furthermore, immunohistochemistry results demonstrated that the live BacHA was able to transduce and express HA in the intestinal epithelial cells in vitro and in vivo. We have demonstrated that recombinant baculovirus with a WSSV ie1 promoter have acted as a vector as well as a protein vaccine and will enable the rapid production of pre-pandemic and pandemic vaccines without any biosafety concerns.
Introduction

The recent outbreaks of H5N1 avian flu and the current pandemic situation with H1N1 swine-origin influenza A virus (S-OIV) are clear indications of the urgent need for effective vaccines against influenza A viruses (31). Preventive and therapeutic measures against influenza A viruses have received a lot of interest and efforts globally, to combat the current pandemic and to prevent such a situation in the future. Currently used vaccines for influenza are mainly administered parenterally and include live attenuated reassortant viruses, conventional inactivated whole viral antigens or split virus vaccines. Although some of these vaccines have proven to be quite effective, the manufacturing of these vaccines involves several technical and safety issues (21). Furthermore, the production of currently available influenza vaccines often requires high level biocontainment facilities- an additional hurdle that limits the advancement of present vaccines.

Vaccines containing purified recombinant viral proteins have recently gained special attention due to their ease of production without any safety concerns (25). Recombinant hemagglutinin (rHA) subunit vaccines produced in baculovirus - insect cell expression system have been extensively tested and evaluated in humans (29, 30). Baculovirus derived rHA subunit vaccines administered parenterally are safe and immunogenic in animals and humans. Along with its success in recombinant protein vaccines, baculovirus surface display technology allows us to present large complex proteins on the baculovirus envelope in its native antigenic conformation resulting in good stability and longer half-life in the host (18, 14, 8).

Along with suitable antigen, route of administration of the vaccine has a profound effect in controlling mucosally acquired infection like influenza. Vaccination via mucosal route stimulates both systemic and mucosal immune
response (16). Oral and intranasal vaccines are the two main options for mucosal administration. Intranasal vaccines would have a detrimental effect to persons with asthma, reactive airway disease and other chronic pulmonary or cardiovascular disorders (4). Oral vaccines therefore seem to be the safest alternative (13). Moreover, there is evidence to prove the ability of oral vaccination to prevent infection of the lungs (23) and cause transcytosis of the molecule across the cells into the circulation (24).

In this report, we describe the construction of recombinant baculovirus under the immediate early promoter 1 derived from the White Spot Syndrome Virus (WSSV) genome, which enables the expression of hemagglutinin at the early stage of infection in insect cells, thereby enhancing the display of HA on the baculovirus envelope. Incorporation of more HA into the budding baculovirus particles would improve their efficacy as immunogens. We have studied the efficacy of WSSV ie1 based BacHA as an oral vaccine in a mouse model of infection. We have also assessed their efficacy with recombinant Cholera Toxin B (rCTB) as a mucosal adjuvant. This strategy will enable rapid production of pre-pandemic vaccines with minimal infrastructure across the world, alleviating the need for high bio-safety facilities, risky inactivation of virulent viruses and meticulous protein purification procedures.

Materials and Methods

Influenza viruses.

The highly pathogenic influenza A Human H5N1 viruses from clade 2.1 A/Indonesia/CDC/669/Indonesia/2006 and A/Indonesia/CDC/594/2006 were obtained from the Ministry of Health (MOH), Republic of Indonesia. The H5N1 viruses from different phylogenetic clades were rescued by Reverse Genetics (36). 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) and clade 8.0 (A/chicken/Henan/12/2004) were synthesized (GenScript, USA) 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 (20). 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). Stock viruses were propagated in the allantoic cavity and virus content was determined by standard hemagglutination (HA) assay as described previously (33). All experiments with highly pathogenic viruses were conducted in a biosafety level 3 (BSL-3) containment facility, in compliance with CDC/NIH and WHO recommendations (15). Recombinant cholera toxin B subunit (rCTB) was provided by Shanghai United Cell Biotechnology Co., Ltd. (Shanghai, P.R.China).

**Generation of recombinant baculovirus vaccine.**

For the construction of recombinant baculovirus Bac-HA, the full length ORF of HA gene (CDC/669/Indonesia/06 and CDC/594/Indonesia/06) was amplified and inserted into pFASTBacHT A (Invitrogen, San Diego, CA, USA) using Rsal and Hind III restriction sites. The ie1 promoter was amplified from WSSV DNA using the primers WSSVie1F-5’-CCTACGTATCAATTTTATGTGGCTAATGGAGA-3’ and WSSVie1R-5’-CGCGTCGACCTTGAGTGGAGAGAGCTAGTTATAA-3’ and then inserted into pFASTBacHT A using AccI and Rsal restriction sites.

For the generation of recombinant baculoviruses the constructs were integrated into the baculovirus genome within DH10Bac™ (Invitrogen, USA) through
site-specific transposition using Bac-To-Bac system (Invitrogen, USA) as described before (19). The recombinant bacmids were then transfected into Sf9-cells and the budded virus particles released into the media were harvested at 4 days post transfection.

Immunofluorescence assay to detect the expression of HA in insect cells.

To detect the Immunofluorescence signals, Sf-9 cells were infected with Bac-HA and the cells were fixed at 48 h post infection as described previously (20). The fixed cells were then incubated with guinea pig anti HA polyclonal antibody at a dilution of 1:100 for 1 h at 37 °C. FITC-conjugated rabbit anti-guinea pig (Dako Cytomation, Denmark) at a dilution of 1:100 was subsequently incubated with the cells for 1 h. The fluorescence signal was detected with an inverted fluorescence microscope (Olympus, UK) and the images were captured by a digital imaging system (Nikon, USA).

Characterization of Baculovirus displaying influenza HA.

The viral titres were determined by plaque assay and the virus particles were purified by two rounds of sucrose gradient ultracentrifugation following the standard protocols (17). Purified recombinant baculovirus in phosphate buffer saline (PBS, pH 7.4) were then mixed with Laemmli sample buffer and resolved by 12% SDS-PAGE. Fractions containing purified baculovirus were then transferred onto nitrocellulose membrane and blocked with 5% non-fat milk in PBST (1x 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 (Dako, Denmark) for 1 h at room temperature. The
membrane was washed and developed by incubation with 3,3'-diaminobenzidine (DAB) and hydrogen peroxide (6). The vaccine was then prepared based on the Hemagglutination titer (HA) of log^2. The inactivated BacHA vaccine was prepared by treating baculovirus displaying HA with Binary ethylenimine (BEI) as described previously (22). The complete loss of infectivity of the inactivated BacHA was determined by inoculation into Sf9 cell monolayer and observation of cytopathic effects for at least 7 days.

**Uptake of recombinant baculovirus by human intestinal cells in vitro**

Human colorectal carcinoma (HCT 116) cells were maintained in McCoy’s 5A modified medium (Cat. No. M4892, Sigma, USA) supplemented with 10 % FBS and seeded in flat bottom 24-well plates on the day of experiment. The cells were incubated with BacHA for 20 h and stained with anti-HA monoclonal antibody, followed by FITC-conjugated goat anti-mouse immunoglobulin (Dako Cytomation, Denmark) (20). In addition, Vero cells maintained in DMEM medium (supplemented with 10 % FBS) were also incubated with BacHA and used as a reference control.

**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. Prior to immunization all mice were starved for 2 h, otherwise food and water were supplied ad libitum. Thirty mice per each experimental group (n=30/group) were immunized intragastrically by oral gavage on days 0, 7 and 21 with 200 µl containing log^2 HA titer of inactivated or live recombinant baculovirus vaccine suspended in phosphate buffered saline (PBS), pH 7.4, either adjuvanted with 10 µg rCTB or unadjuvanted. Six
mice from each experimental group were sacrificed on day 14, 28 and 42, and serum
and intestinal lavage fluids were collected as described previously (32). Briefly, the
small intestine from each mouse was cut into 4–5 cm pieces and transferred to a glass
tube. After addition of 1.0 ml of PBS, the tubes were vortexed gently for 30 s and
centrifuged at 5000 rpm for 10 min.

All animal experiments were carried out in accordance with the Guides 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 of the Temasek Life Sciences Laboratory, National University of
Singapore, Singapore.

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

The HA specific serum IgG antibody titer and the HA specific intestinal
mucosal IgA levels were tested separately against purified rHA0 (Protein Sciences
Corporation CT, USA) antigen by indirect enzyme-linked immunosorbent assay
according to the method previously described (3). In brief, microtiter well ELISA
plates were coated with purified recombinant H5HA in coating buffer (0.1mol/L
carbonate/bicarbonate, pH 9.6). Samples of test serum were diluted serially two fold
in 3% non-fat dry milk/PBS containing 0.05 % Tween 20 (PBS-T) and mucosal wash
were diluted directly 1:20. Then, the colour development was visualized by adding
goat anti-mouse IgG (Sigma, USA) 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, USA). The absorbance was measured at 450 nm
using a microwell plate reader.

Hemagglutination inhibition assay.
Hemagglutination inhibition assays were performed as described previously (33). Receptor destroying enzyme (RDE) treated (2) sera were serially diluted (2-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% chicken RBCs and incubation at room temperature for 40 min.

Microneutralization assay.

The microneutralization test was performed according to the protocol previously described (27). Briefly, MDCK cells were seeded in 96-well culture plates and cultured at 37°C to form a monolayer. Serial two fold dilutions of heat inactivated (56°C for 45 min.) serum samples were mixed separately with 100 TCID$_{50}$ of H5N1 strain and incubated at room temperature for 1h, and the mixtures were added to monolayer of MDCK cells in triplicate wells. The neutralizing titers of mouse antiserum that completely prevented any cytopathic effect at reciprocal dilutions were calculated.

Immunohistochemistry.

The mice were sacrificed on day 28 and the intestine sample was collected in 10% (wt/vol) buffered formalin and embedded in paraffin, and sectioned. The sections were then de-paraffinized using Histo-choice (Amersco, USA) and rehydrated in sequentially graduated ethanol baths. The sections were treated with trypsin (0.1% w/v in PBS) for 10 min and washed twice with PBS-Tween 20 (0.01% v/v with PBS). Slides were blocked in 0.3% non fat milk in PBS for 30 min followed by incubation with guinea pig anti HA polyclonal antibody at a dilution of 1:100 for 1 h at 37°C. FITC-conjugated rabbit anti-guinea pig (DakoCytomation, Denmark) at a dilution of 1:100 was subsequently incubated with the cells for 1 h. The fluorescence signal was
detected with an inverted fluorescence microscope (Olympus, UK) and the images were captured by a digital imaging system (Nikon, USA).

**Disease challenge test against influenza H5N1 virus infection.**

The efficacy of the vaccine was assessed by host challenge against HPAI H5N1 influenza strains. Twenty-one days after final vaccination, mice were transferred into animal BSL3 containment facility. Six mice per group were challenged intranasally with 5MLD50 (Mouse lethal dose 50%) of homologous (CDC/669/Indonesia/06 clade 2.1) and heterologous (Vietnam/1203/2004 clade 1.0) HPAI H5N1 strains. Fifty percent mouse lethal dose (MLD50) of the influenza virus required for intranasal challenge experiments was predetermined. To determine the effect of adjuvant efficacy, animals immunized with vaccines without adjuvant or only with rCTB were also maintained as control groups. Mice were observed daily to monitor body weight and mortality. Monitoring continued until all animals died or until day 14 after challenge. For histopathology, a lung sample was collected in 10% (wt/vol) buffered formalin solution, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin and were analyzed for pathology.

**Statistical analysis.**

The data are expressed as arithmetic mean ± standard Deviation (SD). 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.
Results

Structural and antigenic conformation of HA0 in insect cells and baculovirus envelope.

The indirect immunofluorescence assay revealed that HA0 expressed by the recombinant baculovirus was able to successfully translocate to the plasma membrane of infected insect cells (Fig. 1A). SDS PAGE analysis of budded baculovirus particles from sucrose gradient purification revealed that components of purified baculovirus particles were abundantly present in the 3rd fraction of the gradient (Fig. 1B). Western blot analysis of the fraction containing purified baculovirus indicated that baculovirus surface displayed HA0 was able to sustain its antigenic conformation and authentic cleavage (Fig. 1C).

Baculovirus-mediated expression of influenza HA in human intestinal cells in vitro.

The ability of WSSV ie1 promoter based BacHA to transduce and express HA in human colorectal carcinoma was evaluated in vitro. Indirect immunofluorescence assay demonstrated that live BacHA was able to successfully transduce intestinal epithelial cells and the expression of HA was effectively driven by the WSSV ie1 promoter (Fig. 2A). In addition, BacHA was also able transduce Vero cells in vitro(Fig. 2C). Intestinal cells incubated with inactivated BacHA did not show any positive fluorescence signal compared to that of live BacHA in both cell lines (Fig. 2B & 2D).

Systemic antibody responses to the oral vaccination.
Indirect ELISA was performed to determine the HA-specific serum IgG titers. The groups of mice immunized orally with live BacHA showed significantly ($P<0.001$) enhanced HA specific IgG titers when compared to the inactivated BacHA (Fig. 3A). However, the presence of rCTB adjuvant along with the BacHA, either live or inactivated did not show any significant improvement in antibody titers, when compared with unadjuvanted BacHA (Fig. 3A). The hemagglutination inhibition titers of the sera were also measured. The HI titers also showed a similar trend with no significant difference between the groups orally immunized with the vaccine in the presence or absence of the adjuvant. However the HI titers in the mice immunized with live BacHA titers were significantly ($P<0.001$) higher when compared to the inactivated BacHA (Fig. 3B).

**Mucosal immune responses to the oral vaccination.**

Indirect ELISA was done to determine the HA-specific mucosal IgA levels. The mice immunized with live BacHA showed significantly ($P<0.001$) higher mucosal IgA levels when compared to the mice immunized with inactivated BacHA (Fig. 4). However, the presence of the adjuvant rCTB did not show any increase or decrease in the IgA levels in both the cases (Fig. 4).

**Serum cross-clade neutralizing antibody titer to the oral vaccination.**

The serum neutralizing antibody titer against 100 TCID$_{50}$ of different clades of H5N1 strains on day 42 showed that vaccination with live BacHA alone or the presence of rCTB significantly neutralized ($P<0.001$) viruses from clade 1.0, clade 2.1 (circulating strain from 2007) and clade 8.0 compared with inactivated BacHA alone or in the presence of the adjuvant (Fig. 5). However, the presence of the adjuvant rCTB groups does not influence the neutralizing antibody titers when
compared with unadjuvanted vaccination groups administered with live BacHA and inactivated BacHA (Fig. 5).

**Baculovirus transduction of intestinal epithelial cells in vivo.**

To examine the ability of WSSV ie1 based live baculovirus to mediate gene transduction in the intestinal lumen following oral vaccination, HA expression in the epithelial cells of intestinal villi was examined by immunohistochemistry. The results revealed that live baculovirus was able to transduce HA gene into the epithelial cells of intestinal villi (Fig. 6A). However, inactivated form of baculovirus did not show any immunofluorescence signal (Fig. 6B) and appeared similar to unvaccinated control group (Fig. 6C).

**Challenge studies after oral vaccination.**

Three weeks after final immunization, all groups of mice were challenged intranasally with 5 MLD50 of HPAI H5N1 strains from clade 1.0 or clade 2.1. Groups of mice immunized with either live BacHA alone or in the presence of rCTB lost upto 9% of their original body weight by day 5 or 6 after challenge (Fig. 7C) but provided 100% protection against both clade 1 and clade 2.1 viruses (Fig. 7A & 7B). Moreover, the group of mice that co-administered with live BacHA and rCTB regained their body weight more rapidly (within 6 days) when compared to the mice that immunized with unadjuvanted BacHA, which regained gradually only about 6-7% of the lost body weight on day 14 after challenge. However, mice co-administered with inactivated BacHA and rCTB showed about 14% loss of bodyweight (within 5 days) and provided only 49.9%, 33.3% against clade 2.1 and clade 1.0 respectively (Fig. 7A & 7B). The survived mice from this group gradually regained only about 9% of
Moreover, mice vaccinated with inactivated BacHA alone showed a significant ($P < 0.001$) loss in body weight of up to 23 %, when compared to mice immunized with inactivated BacHA and rCTB on day 5 post challenge. Only 33.3 % of inactivated BacHA immunized mice survived after challenge with H5N1 virus (Fig. 7C). Mice vaccinated with wild – type baculovirus were able to provide only 16.33% protection against viral challenge (Fig. 7A).

**Histopathology.** Histopathology studies were performed in the mice vaccinated and challenged with clade 2.1 virus. On day 6 post infection, lungs of untreated mice had pulmonary lesions consisting of moderate to severe necrotizing bronchitis and moderate to severe histiocytic alveolitis with associated pulmonary edema (Fig 8C). The uninfected mice lacked lesions in the lungs (Fig. 8D). Mice vaccinated with live BacHA had only minimal bronchitis (8A) while mice vaccinated with inactivated BacHA had moderate bronchitis (8B).

**Discussion**

A recombinant baculovirus with the immediate early promoter 1 of WSSV was constructed to facilitate high level expression of influenza H5 hemagglutinin in both insect and mammalian cells. The nature of ie1 as an immediate early promoter supports the protein expression at the early phase of the baculoviral lifecycle, resulting in enhanced display of functional hemagglutinin on the baculovirus envelope. HA displayed on the baculovirus surface has retained its native structure as evidenced by the hemagglutination activity and authentic cleavage of HA0 into HA1 and HA2. Earlier, Treanor et al. (29) reported that parenteral immunizations of influenza HA expressed in insect cells are safe and immunogenic in humans.
However, most studies have only attempted to investigate the efficacy of HA subunit derived baculovirus-insect cell expression system as a vaccine for influenza virus. Since recombinant HA proteins expressed in insect cells tend to form monomers (26), it is reasonable to speculate that it may lead to suboptimal immunogenicity as HA is not being presented in its native trimeric conformation. In fact, Wei et al. (34) demonstrated that oligomeric recombinant HA elicited the strongest immune response in mice compared with that of HA monomer.

Baculovirus surface display technology enables the presentation of large complex proteins in their functional conformation. As the oligomerization is required for efficient transport of the HA proteins to the host cell membrane (5), a pre-requisite for the baculovirus to acquire the protein, it is presumed that HA displayed on the baculovirus surface should have been presented in their oligomeric forms. Hence we attempted to use this baculovirus displaying HA as an oral vaccine candidate against H5N1 infection in mice. The live BacHA was able to induce both systemic and mucosal immune response in the orally vaccinated mice as indicated by the high level of HA specific IgG and IgA antibodies respectively. Interestingly, mice vaccinated with inactivated BacHA were able to induce only low level of immune responses compared to live BacHA. The differences between the immune responses of the mice after live BacHA and inactivated BacHA vaccinations could be mainly due to two factors. Firstly, HA displayed on the live baculovirus would have retained its functional oligomeric conformation resulting in better immunogenicity than inactivated baculovirus. Secondly, native HA could have played a role in binding to the receptors expressed in the intestinal epithelial cell membrane resulting in gene delivery and stimulation of cell-mediated immune response.
Serum hemagglutination inhibition assay and microneutralization assays revealed that live BacHA is superior compared to that of inactivated BacHA vaccine, further suggesting that structural conformation of HA indeed has some effect on its immunogenicity. Serum neutralization efficiency against 100 TCID$_{50}$ of heterologous H5N1 strains from different clades revealed that BacHA vaccination induced significantly higher virus neutralization titers against H5N1 strains from clade 2.1 (circulating strain from 2007). This observation shows the efficacy of the live BacHA vaccine against the genetic drift from 2006 to 2007 in clade 2.1 Indonesian strains. Further, the BacHA also efficiently neutralized, viruses from clade 1.0, clade 2.3 and clade 8.0 compared with inactivated BacHA. This strong cross clade immunity could be due to better affinity and avidity of the antibody response generated against conserved epitopes (3, 12). This is remarkable, as cross-clade viral neutralization is indicative of the critical ability of the vaccine in limiting the evolution of escape mutants by mutation and reassortment. However, further experiments need to be done to better understand the nature of the strong cross clade protection induced by live BacHA vaccination. Our previous observation in mice vaccinated through intranasal route indicated that baculovirus surface-displayed HA efficiently enhanced both systemic and mucosal immune responses compared to inactivated whole H5N1 viral vaccine. Moreover, rCTB containing Bac-HA elicited higher level mucosal and systemic immune responses in a mouse model (19). However in the present study, we did not observe any increase in the antibody responses in the mice orally co-administered with rCTB and BacHA, and the reason why rCTB were not effective is unclear at the moment.

To evaluate the protective efficacy of BacHA vaccines, vaccinated mice were challenged with both homologous and heterologous H5N1 strains. One hundred
percent survival rate was obtained with the group vaccinated with live BacHA with or without rCTB. Interestingly, mice co-administered with live BacHA and rCTB regained their body weight more rapidly compared to those administered with only live BacHA. Further, mice vaccinated with inactivated BacHA resulted only in 33.3% survival rate and adjuvanted with rCTB provided at least 49.9% against homologous virus. Moreover, co-administration of inactivated BacHA and rCTB showed about 14% loss of bodyweight compared ($P<0.001$) to 23% of loss in body weight with unadjuvanted BacHA.

Though the HA specific antibody response is lower with inactivated BacHA compared to its live counterpart, mice vaccinated with inactivated BacHA were also able to provide moderate protective immunity against 5MLD50 of H5N1 viral infection. Earlier reports have shown that intranasal immunization with wild-type baculovirus alone provides sufficient protection from the H1N1 influenza lethal challenge (1). This was ascribed to the recognition of baculoviral envelope protein gp64 by the TLR9 molecule and thus activating the innate immune response (9). Baculovirus is also known to stimulate mammalian cells to secrete IFN cytokines and confer in vivo protection of mice from encephalomyocarditis virus infection (7). Partial protection of mice obtained with inactivated BacHA in this study supports the previous findings that baculovirus can trigger innate antiviral mechanism in the mammalian system.

Baculovirus are able to transduce several mammalian cells and mediate gene transfer in vitro (11). In the present study, uptake of recombinant baculovirus by human intestinal epithelial cells in vitro was confirmed and the expression of HA by
WSSV ie1 promoter was verified. Moreover, reports have demonstrated that recombinant baculoviruses were also able to deliver the genes of interest in vivo in animal models. Tani et al. (28) demonstrated that Vesicular Stomatitis Virus Glycoprotein-modified baculovirus was able to transduce a reporter gene into the cerebral cortex and testis of mice by direct inoculation in vivo. Furthermore, intravitreal injection of baculovirus caused expression of vascular endothelial growth factor in the inner retina, photorecetor cells and in retinal pigment epithelium cells of rabbit eye (10). In the present study, we have also evaluated the potential of live BacHA to transduce the intestinal epithelial cells of orally immunized mice. Immunohistochemical analysis revealed that WSSV ie1 based baculovirus was able to express HA in the epithelial cells of vaccinated mice intestine. Our results suggest that baculovirus have acted as a vectored as well as a protein vaccine against the H5N1 infection.

In summary, the baculovirus surface displayed hemagglutinin vaccine is efficacious in inducing mucosal immune response as well as systemic immune response and does not require either sophisticated biocontainment infrastructure or downstream purification processes for mass production of the vaccine. In addition, the HA of any given influenza virus isolate could be converted into an efficient vaccine with this technology, in a short period of time. Oral vaccination is considered to be a highly desirable form of vaccination, being non invasive, pain-free and self administrable with improved logistics and good immunization coverage. Despite the recent attention towards intranasal administration, oral vaccination is still considered the best approach to increase patient compliance. Parameters such as ease of use, affordability, needle-less administration and mass coverage during pre-pandemic or pandemic situation makes oral vaccination an attractive option. Considering the above
facts, we conclude that the baculovirus surface displayed hemagglutinin vaccine could be an ideal choice for a pandemic or pre-pandemic influenza vaccine.

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REFERENCES


Figure Legends

Figure 1. Characterization of BacHA (A) Indirect immunofluorescence assay with insect cells infected with recombinant baculovirus expressing H5 HA0. Infected cells were fixed and stained with guinea pig anti-HA antibody and rabbit anti-guinea pig FITC. (B) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of purified baculoviruses. Presence of major capsid protein (VP39) and envelope protein (GP64) of baculovirus and influenza HA in the purified sucrose gradient fraction. Lane 1: Pre – stained protein marker, Lane 2: Infected cell culture supernatant, Lane 3, 4 & 5: 1st 2nd & 3rd fraction from Sucrose density gradient purification respectively. (C) and Western blot: Antigenic conformation of HA incorporated into purified baculoviruses (Fraction 3) was probed using anti-HA monoclonal antibody.

Figure 2. Transduction and expression of HA in human intestinal epithelial cells by WSSV ie1 based BacHA. HCT116 cells were incubated with BacHA at a multiplicity of infection (MOI) of 200 and stained with anti HA monoclonal antibody followed by FITC conjugated secondary antibody at 20 hours post infection. Vero cells incubated BacHA at same MOI was used as a reference control. (A) Live BacHA transduced HCT 116 cells. (B) HCT cells incubated with inactivated BacHA. (C) Live BacHA transduced Vero cells. (D) Vero cells incubated with inactivated BacHA.

Figure 3. Measurement of systemic immune response. Groups of mice (n=6) were orally vaccinated three times on days 0, 7 and 21 with 200 µl containing 8 log 2 HA titer of inactivated or live recombinant baculovirus adjuvanted with or with out 10 µg
rCTB. (A) HA specific IgG antibody titers in the serum by indirect ELISA. (B) Serum hemagglutination inhibition titer. Each point represents the arithmetic mean value (n=6) ± SD. (** p<0.001).

Fig 4. Measurement of mucosal anti-HA specific IgA antibody levels by indirect ELISA. Groups of mice (n=6) were orally vaccinated three times on days 0, 7 and 21 with 200 µl containing 8log 2 HA titer of inactivated or live recombinant baculovirus adjuvanted with or without 10 µg rCTB. Each point represents the arithmetic mean value (n=6) ± SD (* p<0.05, ** p<0.02, *** p<0.001).

Fig 5. Cross clade serum microneutralization in mice immunized orally with log 2⁸ titer of inactivated or live recombinant baculovirus with or without 10 µg rCTB. The viruses from clade 1.0 (A/Vietnam/1203/2004), clade 2.1 (A/Indonesia/CDC1031/2007), clade 2.3 (A/chicken/Nongkhai/NIAH400802/2007) and clade 8.0 (A/chicken/Henan/12/2004) were used for this study. The sera from the day of peak response, day 21 after the final immunization, were used for the assay. Each point represents the arithmetic mean value (n=6) ± SE. (* p<0.05, ** p<0.02, *** p<0.001).

Fig 6. Baculovirus transduction of the mice intestinal epithelial cells in vivo. One week after the third immunization, intestinal tissue samples were embedded in paraffin and sectioned. Immunohistochemical staining carried out using a guinea pig anti-HA antibody and rabbit anti-guinea pig FITC. Intestinal villi of mice orally vaccinated with (A) live baculovirus, (B) inactivated baculovirus and (C) PBS.
Fig 7. Protection of mice from lethal H5N1 viral challenge. Groups of mice (n=6) were orally vaccinated three times on days 0, 7 and 21 with 200 µl containing 8log 2 HA titer of inactivated or live recombinant baculovirus adjuvanted with or without 10 µg rCTB. Wild type baculovirus with a dose equivalent to BacHA (based on the viral titers determined by plaque assay) served as a negative control. Three weeks after the final vaccination, mice were intranasally infected with 5MLD50 (Mouse lethal dose 50%) of (A) homologous (CDC/669/Indonesia/06 clade 2.1) and (B) 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. (C) The group of mice challenged with Vietnam/1203/2004 clade 1.0 was also monitored for weight loss throughout a 14 day observation period. The results are expressed in terms of percent body weight (at the beginning of the trial).

Fig 8. Photomicrographs of hematoxylin and eosin stained lung sections of mice challenged with Clade 2.1 H5N1 virus at 6 days post challenge. (A) mice vaccinated with live BacHA, (B) mice vaccinated with inactivated BacHA, (C) unvaccinated mice challenged with virus and (D) normal morphology seen in uninfected mice.
Figure 1A

Figure 2

1B

Kda 1 2 3 4 5
175 HA0 (70 kda)
83 GP64 (64 kda)
62 HA1 (42 kda)
47 VP39 (39 kda)
32
25

1C

HA0 (70 kda)
HA1 (42 kda)
Figure 3
A B
C D
Days pre/post immunization

Serum HA specific IgG titer

Live BacHA+rCTB
Live BacHA
Inactive BacHA+rCTB
Inactive BacHA
Only rCTB

2nd 1st 3rd Immunization

Figure 4
Figure 5

Days pre/post immunization

O.D at 450 nm

0.0
0.2
0.4
0.6
0.8
1.0

Live BacHA+rCTB
Live BacHA only
Inactivated BacHA+rCTB
Inactivated BacHA only
rCTB only
Control

***
***
***
***
***
***
Figure 6
Figure 7

A

B

C

Days Post challenge

Percent Survival

Live BacIA+CTB
Live BacIA
Inactive BacIA+CTB
Inactive BacIA
Only CTB
Wr Baculovirus
Control

Days Post challenge

Percent Survival

Live BacIA+CTB
Live BacIA
Inactive BacIA+CTB
Inactive BacIA
Only CTB
Control

Days post challenge

Percent body weight

Live BacIA+CTB
Live BacIA
Inactive BacIA+CTB
Inactive BacIA
Only CTB
Control

# #

***

A B C
Figure 8