Recombinant baculovirus displayed vaccine
A novel tool for the development of a cross-protective influenza H5N1 vaccine

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The rapid evolution of new sublineages of H5N1 influenza in Asia poses the greatest challenge in vaccine development for pre-pandemic preparedness. To overcome the antigenic diversity of H5N1 strains, multiple vaccine strains can be designed based on the distribution of neutralizing epitopes in the globular head of H5 hemagglutinin (HA). Recently, we selected two different HAs of H5N1 strains based on the neutralizing epitopes and reactivity with different neutralizing antibodies. The HAs of selected vaccine strains were individually expressed on the baculovirus envelope (bivalent-BacHA) with its native antigenic configuration. Further, oral delivery of live bivalent-BacHA elicited broadly reactive humoral, mucosal and cell-mediated immune responses and showed complete protection against antigenically distinct H5N1 strains in mice. The strategy for the vaccine strain selection, vaccine design and route of administration will provide an idea for development of a widely protective vaccine against highly pathogenic H5N1 for pre-pandemic preparedness.

Introduction
Continuous outbreaks of highly pathogenic H5N1 avian flu in Asia and a current situation of new avian flu in China are increasing the threat of the next influenza pandemic. Since April 26, 2013, the World Health Organization confirmed 628 human cases of H5N1 infection with 374 deaths.1 Vaccination against influenza A viruses is the first and the most important step in controlling the spread of the pathogen. All currently available influenza H5N1 vaccines induce protective immunity only toward vaccine-specific strains or within the phylogenetic type/subtypes of H5N1 strains. However, influenza H5N1 strains already evolved and antigenically distinguished into 10 different types and numerous subtypes, which hindered the development of effective vaccines. Hence, a broad cross-protective vaccine could induce some degree of cross-protective immunity against future pandemic H5N1 strain. Several strategies have been evaluated in an effort to increase a cross-protective efficacy of the H5N1 vaccine. Our strategy for the induction of cross-protective efficacy of the vaccine involved three elements: selection of vaccine strains to achieve broad cross-reactive immunity, utilization of vector based efficient antigen delivery and route of administration of the vaccine candidates.

Selection of Vaccine Strains
The selection of vaccine strains based on the distribution of major neutralizing epitopes of hemagglutinin (HA) is the novel way of vaccine selection.2 The major neutralizing epitopes present in the globular head of hemagglutinin (HA) are the principal determinants of protective immunity to influenza virus. Variations in these neutralizing epitopes may render current H5N1 vaccines不合格 for the prevention of heterologous type of H5N1 strains. Hence, distribution pattern of neutralizing epitopes among H5N1 subtype could help in the design
of broadly protective vaccine. Previously, the neutralizing conformational epitopes of hemagglutinin were mapped by the characterization of escape mutants with neutralizing monoclonal antibodies. The neutralizing epitopes located at amino acids 136 to 143 in the 140s loop, amino acids 151–156 in the 150s loop and amino acid at position 189 on of HA1 (H5 numbering, excluding signal peptide) are located within the receptor binding site (Table 1). Inclusion of two or more vaccine strains based on the neutralizing epitopes would cover the variations in the major neutralizing epitopes of most H5 subtype.

We selected two different H5N1 vaccine strains A/Indonesia/CDC669/06 (clade 2.1.3) and A/Anhui/1/05 (clade 2.3.4), characterized by the neutralizing epitopes of HA and sufficient antigenic difference complemented to one another. The reactivity of those vaccine strains with neutralizing antibodies were revealed that neutralizing monoclonal antibody (n-mAb) specific to a conformational epitope (positions 155 Ser and 189 Arg) of A/Indonesia/CDC669/06 H5N1 strain and did not react with A/Anhui/1/05 H5N1 strain. In contrast, neutralizing monoclonal antibodies against a conformational epitope (position 155 Asn and 189 Lys) of HA0 binds only with A/Anhui/1/05 H5N1 strain. These patterns of reactivity could be due to change in the amino acid at position 155 or 189 of conformational epitope region of HA (mature H5 numbering) (Table 1). Two antigenic variants at amino acid position 155 (antigenic site B) and 189 (adjacent to receptor binding, antigenic site B) comprise the majority of human H5N1 isolates. Reactivity pattern of n-mAbs with vaccines revealed that two vaccine strains are complemented each other.

**Viral Vector for Vaccine Delivery**

Second, efficient delivery of such selected vaccine component to the host is essential to elicit broad immune responses. Moreover, vaccine production platform requires minimal technical infrastructure feasibility of large scale deployment at any location in the world. However, the current inactivated influenza vaccines require biosafety containment facility, expensive downstream processing and stable supply of specific-pathogen-free eggs. To overcome these obstacles, several approaches have been adopted including the use of non-human viral vectors for delivery of vaccine. Among those, an insect virus baculovirus has evolved as a tool for vaccine vector development. Baculoviruses are non-enveloped viruses having a circular double-stranded DNA genome. Among the numerous baculoviruses, Autographa californica multicapsid Nucleopolyhedrovirus (AcMNPV) is the most well studied and also utilized as efficient vector for the expression of foreign genes in insect cell system. Baculoviral vector has efficient for gene transfer in a wide range of cell types and it has been exploited as a vaccine vector by displaying foreign antigens on its surface.

**Route of Delivery**

Third, along with this suitable vaccine, the route of administration of the vaccine has a profound effect in controlling mucosally-acquired pathogens including influenza. Oral and intranasal vaccinations are the two main options for induction of mucosal immune response. Intranasal administration of recombinant baculovirus expressing HA elicited a high level of HA specific IgA and serum IgG antibody response in mice. Oral vaccination is considered as another option to stimulate mucosal immunity with increased patient compliance. Oral immunization is non-invasive, affordable with logistics less expensive, and practical for mass vaccination. Previously, we demonstrated that oral administration of baculovirus expressed H5HA vaccine induced high level of antigen-specific mucosal, and systemic immune responses, and cross-protective against H5N1 strain in mice.

We generated a broadly protective bivalent-BacHA vaccine by HAs of selected vaccine strains (A/Indonesia/CD669/06 and A/Anhui/1/05) were individually expressed on the baculovirus under the control of an immediate early promoter 1 (ie1) of white spot syndrome virus (WSSV). The immediate-early promoter supports the protein expression at the early phase of the baculoviral life cycle.
resulting in enhanced processing through the Golgi apparatus and incorporation of functional hemagglutinin into the baculovirus envelope. The HA expressed on the baculovirus surface was able to maintain its authentic cleavage and functional hemagglutination activity, similar to the wild-type influenza virus.

**Bivalent-BacHA Vaccine on the Humoral, Cell-Mediated, and/or Mucosal Immunity**

Induction of both systemic and mucosal immunity is widely viewed as essential role in protection against heterologous influenza infection in humans and animals. We have tested the efficiency of bivalent-BacHA on the humoral and mucosal immune responses by oral or subcutaneous immunizations in mice. We found that mice immunized orally with live bivalent-BacHA stimulated both mucosal and systemic immunity as evident by HA-specific mucosal IgA and serum IgG antibody responses, respectively. However, mice immunized subcutaneously with live bivalent-BacHA or adjuvanted with inactive bivalent-BacHA vaccine significantly induced robust systemic immunity but it does not induce efficient mucosal immunity (Table 2). Apart from the humoral and mucosal immunity, efficiency of the vaccine on the cell-mediated immunity is very important for cross-protection when the humoral immune responses are low. Cellular immunity such as subsets of T helper (Th) cells may be distinguished by the pattern of cytokines that they produce. Th1 cells such as interleukin-2 and interferon gamma (IFN-γ) mediate play a critical role in viral clearance whereas Th2 cells are important for humoral responses. Mice immunized either orally or subcutaneously with live bivalent-BacHA produced Th1 response evident by a high number of IFN-γ secreting cells and also promoted Th2 response evident by increased number of IL-4 secreting cells. In contrast, mice subcutaneously immunized with adjuvanted inactive bivalent-BacHA induced only Th2 response. The overall results are consistent with the idea that oral immunization with baculovirus expressing foreign antigens can elicit mucosal, systemic as well as cell-mediated immune responses (Table 2).

**Bivalent-BacHA on the Cross-Protective Immunity against H5N1 Subtype**

Mice vaccinated orally with live bivalent-BacHA showed neutralizing antibody titer against clades 2.1, 2.3, 4, 7, and 9 but did not neutralize efficiently against clade 1 H5N1 strain. Previously, we generated a trivalent-BacHA vaccine by inclusion of clade 1 (A/Vietnam/1203/04) H5N1 strain along with this bivalent vaccine composition and the vaccine study showed that mice subcutaneously with adjuvanted inactive trivalent-BacHA vaccine elicited neutralizing antibody titer against all H5N1 strains including clade 1.7 This might due to solely only on the induction of antibody-mediated immunity by subcutaneous immunization of inactive form of vaccine. Furthermore, mice subcutaneously immunized with live bivalent-BacHA showed complete protection against distinct H5N1 strains (clade 1 and clade 2.2.1.1) whereas mice vaccinated with adjuvanted inactive bivalent-BacHA vaccine showed comparatively less protection against clade 1 (Table 2).

**Table 2. Overall protective immunity of bivalent-BacHA vaccine**

| Route of vaccination | Vaccine and dosage | Humoral immune response | Mucosal antibody response | Cell-mediated immunity | Cross-protective immunity against 5 Mouse lethal dose (percent survival) |
|----------------------|--------------------|------------------------|--------------------------|-----------------------|---------------------------------------------------------------|
|                      |                    | Hamagglutination inhibition titer | Mucosal HA specific IgA antibody level (optical density at 450 nm by ELISA) | (Number of spots in 10⁶ spleenocytes) |                                                                |
|                      |                    |                        |                          |                       |                                                                |
| Gastrointestinal     | 10⁸ PFU of live Bivalent-BacHA | >100                   | >0.8                     | >300                  | >80 100% 100%                                                  |
| Subcutaneous         | 10⁸ PFU of live Bivalent-BacHA | >200                   | 0.2                      | >300                  | >150 100% 100%                                                  |
| Subcutaneous         | 10⁸ PFU of inactive Bivalent-BacHA + Seppic ISA53 adjuvant | >250                   | <0.2                     | 100                   | >200 66.6% 100%                                                  |
| Subcutaneous         | Inactive whole H5N1 vaccine (Indonesia/MDV01)+ Seppic ISA53 adjuvant | >220                   | <0.2                     | <100                  | >150 33.3% 66.6%                                                  |

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adjuvanted inactive monovalent whole viral vaccine (A/Indonesia/CDC669/06) were neutralized against homologous H5N1 strain and inability to protect against heterologous H5N1 challenge (Table 2). Hence, the cross-clade neutralizing efficacy of the bivalent-BacHA vaccine was likely due to efficient coverage of variations within the major neutralizing epitopes (Table 1) and also high affinity and avidity of the antibodies generated against other conserved neutralizing epitopes.8

In summary, the results of cross-clade protective efficacy of the oral immunization of live bivalent-BacHA vaccine were comparable with subcutaneous immunization of adjuvanted inactive trivalent-BacHA in mice.7 However, we should point out some of the limitations of live bivalent-BacHA compared with subcutaneous immunization of inactive trivalent-BacHA vaccine. Although orally or subcutaneously immunized with live bivalent-BacHA vaccine shows complete protection against distinct H5N1 challenge, still shows some clinical symptoms and reduced body weight after the viral challenge. Although oral vaccination of bivalent-BacHA vaccine stimulates mucosal immunity, additional booster dose will be required to reach maximal protection against influenza H5N1 infection when compared with subcutaneous immunization of inactive form of trivalent-BacHA.

Conclusion
Overall study concluded that induction of cell-mediated and mucosal immunity play an important role in recovery and protection during distinct H5N1 infection, especially when protective antibodies titers are low. Baculovirus expressed selected HAs of H5N1 strain induces antigen specific humoral, mucosal, and cell-mediated immunity against distinct H5N1 strains. Also, baculovirus based delivery system is advantageous over other human viral vectors due to low cytotoxicity, inability of virus to replicate in mammalian cells and absence of pre-existing immunity in humans. Furthermore, oral delivery is a viable option which shows increased patient compliance, safety, and ease of administration. The strategy for the vaccine strain selection, vaccine design, and route of administration will provide an idea for development of a broadly protective vaccine against highly pathogenic H5N1 for pre-pandemic preparedness.

Disclosure of Potential Conflicts of Interest
No potential conflict of interest was disclosed.

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