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Subcutaneous Immunization with Baculovirus Surface-Displayed Hemagglutinin of Pandemic H1N1 Influenza A Virus Induces Protective Immunity in Mice

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The protective immunity of baculovirus displaying influenza virus hemagglutinin (BacHA) against influenza 2009 H1N1 virus infection in a murine model was investigated. The results showed that mice vaccinated with live BacHA or an inactive form of adjuvanted BacHA had enhanced specific antibody responses and induced protective immunity against 2009 H1N1 virus infection, suggesting the potential of baculovirus as a live or inactivated vaccine.

The 2009 pandemic influenza H1N1 (2009 H1N1) virus continues to be transmitted among humans worldwide and cause reported laboratory-confirmed human infections, including 18,449 fatalities (15). Vaccination against influenza A viruses is an important step in controlling the spread of the pathogen. However, currently licensed 2009 H1N1 vaccines mainly rely on inactivated viruses produced in embryonated hens’ eggs, the processing of which requires a containment facility, expensive downstream processes, and a stable supply of specific-pathogen-free eggs (8). Moreover, local or systemic allergic reactions to residual egg proteins in the vaccine components can occur for some individuals (3). This warrants the need for simple and rapid production of pandemic vaccines with minimal technical infrastructure at any location in the world. Recently, it has been shown that baculovirus-derived influenza virus surface glycoprotein hemagglutinin (HA), administered parenterally, is safe and induces antibody-mediated protective immunity in both animal and human trials (12, 13). However, recombinant proteins or baculovirus-expressed insect-cell HA proteins may not always retain their antigenic conformation in the vaccine formulation, resulting in poor immunogenicity when used as immunogens in humans (2, 11). Moreover, the low solubility of the hydrophobic HA protein increases the difficulty of purification and thus elevates the overall production costs. To circumvent these issues, a baculovirus surface display system has been developed as a novel tool for vaccine production. Here, we investigated the protective effect of baculovirus surface-displayed HA driven by a white spot syndrome virus (WSSV) immediate early promoter 1 (ie1) as a live or inactive form of vaccine against 2009 pandemic H1N1 influenza virus infection.

A recombinant baculovirus (BacHA) expressing influenza virus hemagglutinin of a 2009 H1N1 strain (A/Singapore/TLL01/2009) under the control of an immediate early promoter 1 of WSSV to facilitate display of functional hemagglutinin on the baculovirus envelope was constructed (7). Briefly, the full-length HA gene from A/Singapore/TLL01/2009 (GenBank accession no. GOQ27164) and the internal six genes from A/Puerto Rico/8/1934 (PR8) was amplified and inserted along with the WSSV ie1 promoter into the pFastBac1 vector, and the constructs were integrated into the baculovirus genome. The recombinant bacmids were then transfected into Sf9 cells (Invitrogen), and the budded virus particles were purified by use of a sucrose gradient (6). For the reference vaccine, a reasortant virus containing the HA and neuraminidase (NA) from A/Singapore/TLL54/2009 H1N1 (GenBank accession no. GOQ27164) and the internal six genes from A/Puerto Rico/8/1934 (PR8) was generated (16). Further, the virus was purified by ultracentrifugation at 100,000 x g for 2 h at 4°C and PFU were quantified by plaque assay. Then, the reverse genetics H1N1 (RG-H1N1) and BacHA viruses were inactivated with binary ethylenimine n(BEI) as described previously by Rueda et al. (9) and King (4). The HA displayed on the baculovirus envelope retained the ability to bind sialic acid receptors on chicken red blood cells in a manner similar to that of wild-type influenza virus (data not shown). Also, the nature of ie1 as an immediate early promoter supports HA protein expression at the early phase of the baculoviral life cycle, resulting in enhanced processing through the Golgi apparatus and incorporation into the baculovirus envelope (5).

Twelve specific-pathogen-free female BALB/c mice (5 to 6 weeks old) per group were immunized subcutaneously two times on days 0 and 28 with 7.7 x 10^7 PFU of live BacHA...
(approximately 128 HA titer) or inactivated BacHA either alone or emulsified with Montanide ISA563 adjuvant (water in oil emulsion; SEPPIC, France). As a reference control, a group of mice was vaccinated similarly with $5 \times 10^7$ PFU of inactivated 2009 RG-H1N1 virus (A/Singapore/TLL54), alone or emulsified with Montanide ISA563 adjuvant. All animal procedures were conducted under institutionally approved protocols.

Serum samples were collected from 10 mice per experimental group on days 14, 28, and 42. Serum HA-specific antibodies were measured by indirect enzyme-linked immunosorbent assay (ELISA) (7), and hemagglutination inhibition (HI) assay was done with the 2009 H1N1 A/Singapore/TLL52 virus (GenBank accession no. GQ527166) (14). The data are expressed as arithmetic means ± standard deviations (SD). One-way analysis of variance (ANOVA) and the Tukey honestly significant difference (HSD) post hoc test were used to determine which groups were significantly different from the rest. The results showed that mice vaccinated with live BacHA had significantly ($P < 0.0001$) enhanced HI titers and HA-specific IgG antibody titers on day 42 compared to those of mice vaccinated with unadjuvanted, inactivated BacHA (Fig. 1A and B). However, mice immunized with the adjuvanted, inactive form of either BacHA or RG-H1N1 had significantly ($P < 0.0001$) enhanced HI titers and HA-specific IgG titers compared to their unadjuvanted counterparts on any day of testing (Fig. 1A and B). The levels of HA-specific serum IgG ($>230$) and hemagglutination inhibition titers ($>225$) induced by inactivated, adjuvanted BacHA were comparable to those induced by the inactivated RG-H1N1 vaccine with the adjuvant.

In addition, a serum microneutralization test against 100 50% tissue culture infective doses (TCID$_{50}$) of the A/Singapore/ON1723/2009 strain was performed on day 42 according to a previously described protocol (10). The results showed that mice vaccinated with the inactivated, adjuvanted BacHA vaccine had significantly ($P < 0.0001$) high-level neutralizing antibody titers compared with mice vaccinated with the unadjuvanted, inactivated counterpart (Fig. 1C). Moreover, administration of the adjuvanted, inactive forms of the BacHA and RG-H1N1 vaccines produced similar neutralizing antibody titers following the primary or secondary vaccinations.

**FIG. 1.** The specific immune responses of mice vaccinated with live baculovirus surface-displayed HA from A/Singapore/TLL01/2009 (BacHA) or inactivated BacHA with an adjuvant. A reference group of mice was vaccinated with an inactivated RG-H1N1 (A/Singapore/TLL54/2009) strain. (A) Serum hemagglutination inhibition titers. Each point represents the arithmetic mean value ($n = 10$) ± SD. ***, $P$ value of $<0.0001$ between live BacHA- and unadjuvanted, inactivated BacHA-vaccinated groups (a) or between adjuvanted, inactivated BacHA- or RG-H1N1-vaccinated mice and unadjuvanted vaccine-receiving counterparts (b). (B) HA-specific IgG antibody titers in serum, determined by indirect ELISA. Each point represents the arithmetic mean value ($n = 10$) ± SD. ***, $P$ value of $<0.0001$ between live BacHA- and unadjuvanted, inactivated BacHA-vaccinated groups (a) or between adjuvanted, inactivated BacHA- and unadjuvanted, inactivated BacHA-vaccinated groups (b). (C) Serum microneutralization titers of vaccine against a pandemic H1N1 (A/Singapore/ON1723/2009) strain. The sera from day 42 (14 days after final immunization) were used for the assay. Each point represents the arithmetic mean value ($n = 10$) ± SD. *, $P < 0.05$; ***, $P < 0.0001$.**
ingly, the live BacHA vaccine efficiently neutralized the 2009 H1N1 strain (A/Singapore/ON1723/2009) compared to the in-active form of unadjuvanted BacHA. This could be in part due to the ability of the live recombinant baculovirus to efficiently transduce the HA gene into the host tissue and/or due to the adjuvant properties of the unmethylated CpG motif present in the baculoviral DNA (1).

The level of protective immunity induced by vaccination was determined by challenging eight mice in each group intranasally with $10^5$ TCID$_{50}$ of 2009 H1N1 (A/Singapore/104/2009) on day 49. All animal challenge experiments were conducted in an animal biosafety level 3 containment facility. While the viral challenge strain did not cause mortality in mice, it caused a rapid decline in the body weights of the unprotected animals, which were reduced by almost 15% on day 6 after the challenge (Fig. 2A). The progression of infection was indicated by various trends of decrease in body weight in the different groups. Mice immunized with either the adjuvanted, inactivated BacHA or RG-H1N1 vaccine showed no decrease in body weight after the challenge (Fig. 2A). Moreover, mice vaccinated with live BacHA had a reduction of only up to 3% of the original body weight, and 6 days after the challenge, the mice rapidly regained their lost body weight. However, mice vaccinated with the unadjuvanted, inactivated BacHA or RG-H1N1 viral vaccine showed a 10% loss of body weight (Fig. 2A). Also, 6 days after the viral challenge, the right and left lungs from two mice were aseptically removed for virus titration and histopathology examination (7), respectively. The lungs of unvaccinated control mice had the highest viral titers (3.6 log$_{10}$ TCID$_{50}$) and had pulmonary lesions indicative of moderate bronchitis and moderate to severe histiocytic alveolitis (Fig. 2C, panel V). Mice vaccinated with unadjuvanted, inactivated BacHA or RG-H1N1 had lower viral titers (2.3 log$_{10}$ TCID$_{50}$) than the unvaccinated group (Fig. 2B). However, mice vaccinated with inactive form of adjuvanted BacHA or RG-H1N1 showed undetectable viral titers (Fig. 2B) and had only normal to minimal bronchitis (Fig. 2C, panel II or III, respectively). Interestingly, mice vaccinated with only live BacHA had an undetectable viral titer in the lungs and had only minimal bronchitis and minimal histiocytic alveolitis (Fig. 2C, panel IV). The overall protective immunity study revealed that mice vaccinated with live BacHA or the inactive form of BacHA adjuvanted with Montanide ISA563 showed complete protection from 2009 pandemic H1N1 (A/Singapore/104/2009) viral infection. Previously, our group has demonstrated the use of baculovirus surface-displayed HA as a mucosal vaccine candidate against H5N1 infection in mice (7).

In summary, the level of immune response and protective immunity obtained with the adjuvanted, inactive form of baculovirus was comparable with live baculovirus, suggesting the
potential of baculovirus as a live or inactivated vaccine. In addition, with this approach, the HA of any given influenza virus isolate can be converted into an efficient vaccine in a short period of time.

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