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Short Communication

Protective immunity elicited by a pseudotyped baculovirus-mediated bivalent H5N1 influenza vaccine

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ABSTRACT

The development of novel H5N1 influenza vaccines to elicit a broad immune response is a priority in veterinary and human public health. In this report, a baculovirus vector was used to construct bivalent recombinant baculovirus vaccine encoding H5N1 influenza virus hemagglutinin proteins (BV-HAs) from clade 2.3.4 and clade 9 influenza viruses. Mice immunized with 5×10^7 IFU BV-HAs developed significantly high levels of H5-specific neutralizing antibodies and cellular immunity that conferred 100% protection against infection with H5N1 influenza viruses. This study suggests that baculovirus-delivered multi-hemagglutinin proteins might serve as a candidate vaccine for the prevention of pre-pandemic and pandemic H5N1 influenza viruses.

Since 2003, repeated outbreaks of highly pathogenic avian influenza (HPAI) H5N1 virus infections have occurred in poultry in eastern Asia and have had significantly devastating effects on the poultry industries of these regions (Li et al., 2004). More importantly, H5N1 influenza virus can be transmitted directly from infected poultry to humans, causing serious respiratory diseases and even fatalities (Abdel-Ghafar et al., 2008). The wide geographic distribution and spread of H5N1 influenza virus in avian species and in humans is unprecedented (Neumann et al., 2010). Together with the ongoing genetic evolution of this virus, H5N1 influenza virus has a high potential for future pandemics. Therefore, it is an urgent need to develop effective vaccines to control the spread of H5N1 influenza virus, and the prevention of transmission of the virus between poultry and humans is a global priority.

In our previous study, baculovirus pseudotyped with vesicular stomatitis virus (VSV) glycoprotein was used as a vector to express the hemagglutinin (HA) protein of HPAI, A/Chicken/Hubei/327/2004 (HB/327). Mice immunized with the resultant recombinant baculovirus (BV-G-HA) developed effective immune responses and afforded complete protection against infection by homologous virus (Wu et al., 2009). However, only moderate protection was provided against an evolutionarily distant strain (unpublished data).

It is well known that the main barrier in influenza virus vaccine development is the high degree of genetic variability of the virus (Chen et al., 2008). For instance, current monovalent inactivated whole virus vaccine could induce a substantial neutralizing antibody response against the homologous strain, but a decreased response to heterologous strains (Ehrlich et al., 2008). One way to minimize the antigenic diversity between the vaccine strain and circulating viruses is to perhaps create a vaccine expressing antigens covering the major circulating virus strains (Prabakaran et al., 2010). To attempt to broaden the protective effect of the BV-G-HA vaccine, we constructed a bivalent recombinant baculovirus vaccine expressing two HA proteins derived from two different HPAI H5N1 viruses, clade 9 and clade 2.3.4 (known as 'Fujian-like' viruses), representing the circulating strain in central China and in southeast Asia in recent years (Li et al., 2010; Zhou et al., 2010; Zou et al., 2009). The protective immune elicited by BV-HAs was evaluated and compared with those induced by a mono-HA-based BV-HA in a mouse challenge model.

To construct the recombinant baculovirus encoding multiple HA proteins from different strains of H5N1 viruses, the HA gene coding sequences from clade 2.3.4 and clade 9 viruses were optimized for mammalian expression and synthesized (GenScript). The synthesized HA gene fragments were inserted into the pFastBac-VSV/G (Wu et al., 2009), under the control of the immediate early promoter of cytomegalovirus (CMV). Recombinant BV-HAs (Fig. 1A) were subsequently generated using the Bac-to-Bac® system (Invitrogen). A recombinant BV-HA expressing the codon-optimized HA gene of clade 2.3.4 was also constructed (Fig. 1A).

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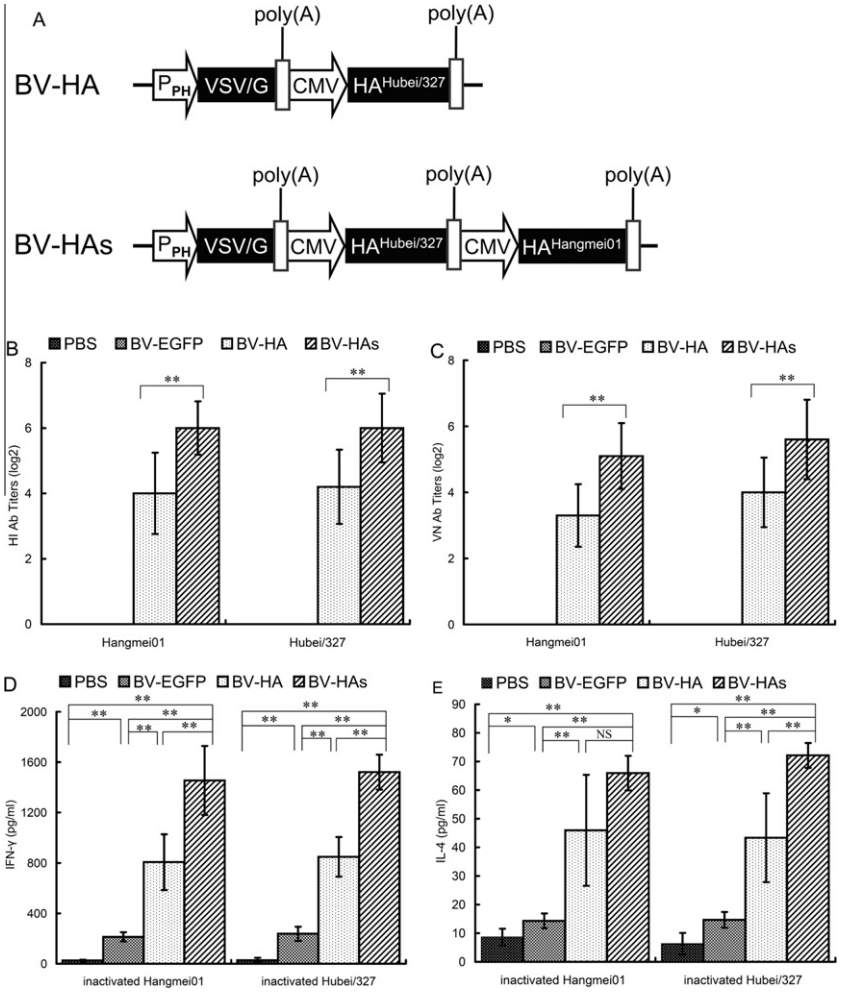

Fig. 1. Humoral and cellular immune responses in mice immunized with BV-HAs and BV-HA. (A) Schematic diagram of recombinant baculoviruses. P_{PH}, the polyhedrin promoter of baculovirus; VSV/G, the glycoprotein of vesicular stomatitis virus; CMV, cytomegalovirus immediate-early promoter/enhancer; poly(A), polyadenylation signal; $HA^{Hubei/327}$, the HA gene encoding the hemagglutinin protein of HB/327; $HA^{Huangmei01}$, the HA gene encoding the hemagglutinin protein of Huangmei01. Serum samples were collected 2 weeks post booster immunization to determine the HI antibody titers (B) and VN antibody titers (C). Splenocytes were isolated and stimulated with 10 μg/ml of inactivated H5N1 influenza virus Hangmei01 or HB/327 for 72 h to determine secretion of IFN-γ (D) and IL-4 (E) by ELISA. All data represent the mean ± SD. Significantly difference was statistically analyzed by one-way analysis of variance (ANOVA) with Turkey's multiple comparison test, *means $P < 0.05$, **means $P < 0.01$, NS means $P > 0.05$ (no statistically significant).

Viruses were purified and titrated as previously described (Wu et al., 2009). The expression of the HA protein was confirmed by western blot (data not shown).

In order to investigate the immunogenicity of recombinant baculoviruses, groups of female, six-week-old BALB/c mice (33 mice per group) were intramuscularly (i.m.) immunized with 5×10^7 infection-forming units (IFU) of BV-HA, BV-HAs or a recombinant baculovirus expressing the enhanced green fluorescent protein (BV-EGFP) (Wu et al., 2009) twice at three-week intervals. Another group of animals were immunized with PBS as a negative control. At 3 and 5 weeks after primary inoculation, virus neutralizing (VN) antibodies and hemagglutination inhibiting (HI) antibodies were detected as previously described (Jiang et al., 2007). All mice immunized with BV-HAs or BV-HA elicited detectable H5-specific ELISA antibodies at 3 weeks after primary immunization, while only a few mice developed low HI and VN antibodies against Hubei/327 or Hangmei01 (data not shown). However, following booster immunization, the mean titers of HI and VN antibodies increased greatly, reaching up to 1:16 to 1:64 (Fig. 1B and C). Compared with the BV-HA, BV-HAs can induce more efficient antibody responses ($P < 0.01$), which may be contributed to the combination of co-expressing two different HA antigens and the high level of expression of these antigens *in vivo*. It is believed that the level of protein expression in an immunized host is positively correlated with the induced immune response (Greenland et al., 2007). Moreover, Western blot and densitometric analysis of band intensity showed that codon optimization of the HA gene and two independent HA expression cassettes greatly improved HA expression in mammalian cells (data not shown).

Apart from serological tests, cellular immune responses were also evaluated after the booster immunization by measuring the expression of IFN-γ and IL-4 in splenocytes as described previously (Wu et al., 2009). Mice immunized with BV-HAs resulted in the highest level of secretion of IFN-γ, which was almost 2-fold higher than that of the BV-HA group ($P < 0.01$) and 40-fold higher than that of the PBS group ($P < 0.01$) (Fig. 1D). As for IL-4 secretion, it was the same pattern to the IFN-γ (Fig. 1E). However, the amount of IL-4 was much lower than that for IFN-γ, suggesting a strong Th1-biased cellular immune response induced by BV-HAs and BV-HA. Furthermore, mice immunized with BV-EGFP also developed a certain level of IFN-γ ($P < 0.05$) and IL-4 ($P < 0.05$) secretion compared to the PBS group, showing that the recombinant baculovirus could elicit a non-specific cellular immune response *in vivo*.

Next, to evaluate the protective immunity elicited by BV-HAs, 2 weeks after secondary immunization, mice were intranasally (i.n.) infected with 1.69×10^6 50% egg infectious dose (EID_{50}) of

HB/327 (50 × 50% mouse lethal doses [MLD_{50}]) or 3.85 × 10^2 EID_{50} of Hangmei01 (50 MLD_{50}). Mice immunized with PBS or BV-EGFP exhibited serious weight loss and signs of illness until to death. However, only moderate and slight weight loss was observed in mice immunized with BV-HA and BV-HAs separately (Fig. 2A and B). Log-rank analysis showed that there was significantly different ($P < 0.05$, by χ^2 test) between BV-HAs-immunized mice (100%) and BV-HA-immunized mice (50%) post-challenge with H5N1 virus from clade 2.3.4 (Fig. 2C), which is heterologous to the BV-HA vaccine. After challenged with H5N1 virus from clade 9, which is homologous to the vaccine BV-HA, mice immunized with BV-HAs provided 100% protection, while 75% survival was observed in mice immunized with BV-HA (Fig. 2D). These results indicated that co-expression of multi-HAs could enhance broad protection against virus challenge.

For further understanding the protection conferred by BV-HAs, six mice per group were sacrificed on day 5 post-challenge, and lung and brain tissues were collected for analysis of viral titers by plaque assay in MDCK cells and histopathological changes (Svetlikova et al., 2010; Wu et al., 2009). As shown in Table 1, significantly reduced viral burdens were found in the lungs and brains of mice immunized with BV-HA, compared with the control groups ($P < 0.01$). However, there were no viruses recovered from the organs of mice immunized with BV-HAs. Additionally, the severities of histopathological changes in these tissues were positively correlated to the virus burdens in these tissues (data not shown). These data indicate that the immune responses induced by bivalent vaccine BV-HAs not only inhibited viral replication in lungs but also systemically blocked migration of viruses from lung to brain.

In the present study, immunization with BV-HAs induced significantly higher humoral and cellular immune responses and conferred complete protection against H5N1 influenza virus from clade 2.3.4 and clade 9, compared with the BV-HA vaccine. However, the immunizing dose used was only 1/20 of that used in our previous study (Wu et al., 2009). These robust immune responses may be attributed to not only the high expression of antigens by BV-HAs *in vivo* but also baculovirus itself possessing a strong adjuvant activity to enhance the immune response against coadministered antigens. It is reported that immunization of mice with ovalbumin plus baculovirus genomic DNA was sufficient to promote humoral and cytotoxic T-lymphocyte responses against co-administered antigens (Hervas-Stubbs et al., 2007). Furthermore, baculovirus was shown to preferentially infect dendritic cells and B cells and promote natural killer cell proliferation *in vivo*, which triggers signaling cascade pathways in these immune competent cells to secrete various cytokines, including interferons (Seo et al., 2002). In addition, recent mechanism studies elucidated that baculovirus has abundant CpG and induces innate immune responses through at least two different signaling pathways, the TLR9/MyD88-dependent endosomal recognition pathway and the DNA-dependent activator of interferon-regulatory factors mediated pathway (Abe et al., 2009).

Table 1
Virus replication in immunized mice challenged with 50 MLD_{50} of H5N1 influenza virus Huangmei01 and HB/327.

Groups	Challenged with Hangmei01		Challenged with Hubei/327	
	Lungs[a]	Brains[a]	Lungs	Brains
PBS	5.01 ± 0.09	5.24 ± 0.08	4.81 ± 0.52	3.99 ± 0.47
BV-EGFP	4.93 ± 1.42	5.55 ± 0.20	4.93 ± 0.99	4.44 ± 0.38
BV-HA	2.99 ± 0.12	3.24 ± 0.19	2.68 ± 0.39	2.13 ± 0.08
BV-HAs	/[b]	/[b]	/	/

Note: Data are presented as mean ± SD, unless otherwise indicated.
[a] The lungs and brains were collected at 5 days post-challenge and then homogenized in 1 ml cold PBS for virus isolation in MDCK cells by serial 10-fold dilutions of samples. Results are presented as mean ± SD for three mice. The viral titers in the tissues were expressed as $\log_{10}$ PFU/ml. The lower limit of virus detection was 2 $\log_{10}$ PFU per 1 ml.
[b] No virus was recovered from the lungs and brains of BV-HAs group.

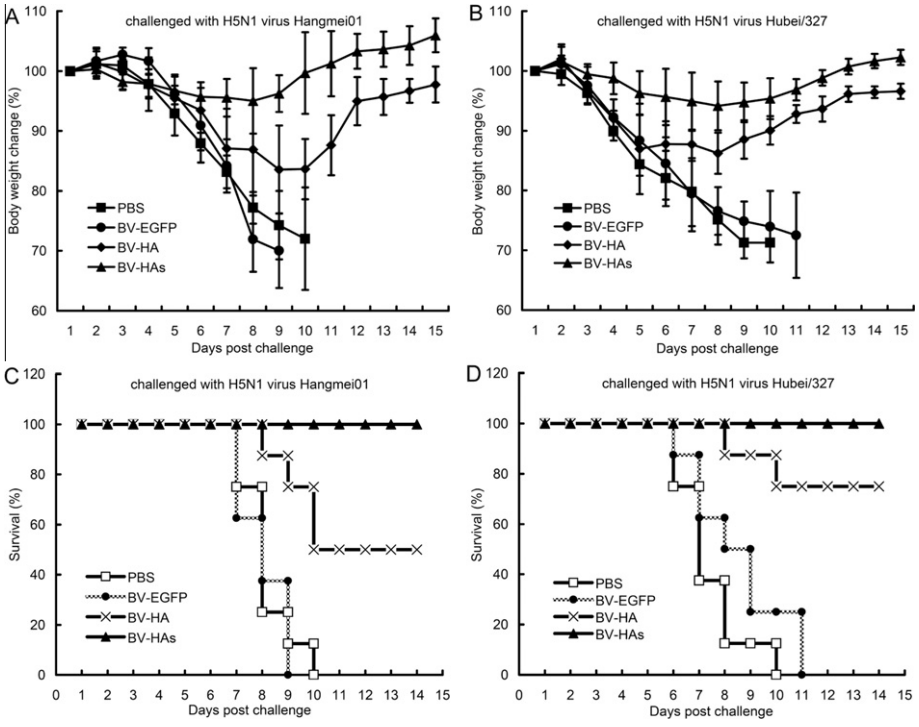

Fig. 2. Protection efficacy elicited with BV-HA and BV-HAs vaccines in mice challenged with 50 MLD_{50} of H5N1 viruses Hangmei01 and Hubei/327. (A and B) Weight loss was evaluated for 14 days after challenge and expressed as mean ± SD of percentage of original body weight. (C and D) Survival curves of the immunized groups. For BV-HA group versus BV-HAs group, $P < 0.05$ post-challenge with Hangmei01 and $P > 0.05$ post-challenged with Hubei/327, by log-rank test.

Baculovirus is an easily manipulable virus that has a large DNA insertion capacity, which makes it readily amenable to modification once a newly circulating strain is identified. Currently, industrial scale production of baculovirus by suspension insect cell cultures is feasible and carries a relatively low cost, although new technological processes to purify the baculovirus should be improved. Furthermore, the absence of pre-existing antibodies to baculovirus makes it provide a highly efficient gene delivery into immune competent cells *in vivo* by direct infection without any replication. These advantages together with the improved immunogenicity of BV-HAs, make it a worthy candidate vaccine for pre-pandemic and pandemic flu and provide new technology for producing influenza vaccines without the requirement of embryonated chicken eggs and biosafety containment.

Conflict of interest

All authors declare no competing interest.

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