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Sublingual Administration of Bacteria-Expressed Influenza Virus Hemagglutinin 1 (HA1) Induces Protection against Infection with 2009 Pandemic H1N1 Influenza Virus

Byoung-Shik Shim[1†], Jung-ah Choi[1†], Ho-Hyun Song[1], Sung-Moo Park[1,2], In Su Cheon[1,2], Ji-Eun Jang[1], Sun Je Woo[1,3], Chung Hwan Cho[1,2], Min-Suk Song[4], Hyemi Kim[5], Kyung Joo Song[5], Jae Myun Lee[5], Suhng Wook Kim[6], Dae Sub Song[7], Young Ki Choi[4], Jae-Ouk Kim[1], Huan Huu Nguyen[1], Dong Wook Kim[8], Young Yil Bahk[9], Cheol-Heui Yun[2], and Man Ki Song[1*]

[1]Laboratory Science Division, International Vaccine Institute, Seoul 151-919, Republic of Korea
[2]Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences, and the Center for Agricultural Biomaterials, and Center for Food Safety and Toxicology, Seoul National University, Seoul 151-921, Republic of Korea
[3]Department of Oral Microbiology and Immunology, Dental Research Institute, and BK21 Program, School of Dentistry, Seoul National University, Seoul 110-749, Republic of Korea
[4]College of Medicine and Medical Research Institute, Chungbuk National University, Cheongju 361-763, Republic of Korea
[5]Brain Korea 21 Project for Medical Sciences, Yonsei University College of Medicine, Seoul 120-752, Republic of Korea
[6]Department of Biomedical Science, College of Health Sciences, Korea University, Seoul 136-703, Republic of Korea
[7]Viral Infectious Disease Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Republic of Korea
[8]Department of Pharmacy, College of Pharmacy, Hanyang University, Kyeonggi-do 426-791, Republic of Korea
[9]Department of Biotechnology, Konkuk University, Chungju 380-701, Republic of Korea

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Influenza viruses are respiratory pathogens that continue to pose a significantly high risk of morbidity and mortality of humans worldwide. Vaccination is one of the most effective strategies for minimizing damages by influenza outbreaks. In addition, rapid development and production of efficient vaccine with convenient administration is required in case of influenza pandemic. In this study, we generated recombinant influenza virus hemagglutinin protein 1 (sHA1) of 2009 pandemic influenza virus as a vaccine candidate using a well-established bacterial expression system and administered it into mice via sublingual (s.l.) route. We found that s.l. immunization with the recombinant sHA1 plus cholera toxin (CT) induced mucosal antibodies as well as systemic antibodies including neutralizing Abs and provided complete protection against infection with pandemic influenza virus A/CA/04/09 (H1N1) in mice. Indeed, the protection efficacy was comparable with that induced by intramuscular (i.m.) immunization route utilized as general administration route of influenza vaccine. These results suggest that s.l. vaccination with the recombinant non-glycosylated HA1 protein offers an alternative strategy to control influenza outbreaks including pandemics.

***Keywords*:** Hemagglutinin, mucosal immune response, non-glycosylation, pandemic, sublingual

Introduction

The 2009 pandemic influenza A (H1N1) virus (2009 H1N1) spread rapidly throughout the world (Igarashi *et al*., 2010) in more than 135 countries (Khan *et al*., 2009). The emergence and rapid spread of pandemic influenza virus challenged the vaccine manufacturers to develop a new strategy for rapid and mass production of influenza vaccines (Aguilar-Yanez *et al*., 2010). Currently, there are two types of influenza vaccines licensed to be used in humans: inactivated vaccines and live-attenuated vaccines (Hai *et al*., 2011). The aim of these vaccines is to induce neutralizing antibodies (nAbs) against the hemagglutinin (HA), which is a key protective mechanism against influenza infection. Both vaccines are produced in embryonic chicken eggs allowing virus propagation (Aguilar-Yanez *et al*., 2010). However, egg-based production is difficult to be scaled up in a short period of time especially under an emergency situation such as pandemic (Reisinger *et al*., 2009).

Bacteria expression system has been an alternative to produce influenza HA-subunit vaccine (Song *et al*., 2008a; Igarashi *et al*., 2010), since it offers no limitations in scaling up to produce large quantity of vaccines in a short period of time (Song *et al*., 2008a). Several studies demonstrated that immunization with a non-glycosylated recombinant vaccine induced protective immunity against influenza viruses (Berhanu *et al*., 2008; Song *et al*., 2008a; Aguilar-Yanez *et al*., 2010), although glycosylation has been considered to be

[†]These authors contributed equally to this work.
*For correspondence. E-mail: mksong@ivi.int; Tel.: +82-2-881-1404; Fax: +82-2-881-1410
Abbreviations. Abs, antibodies; ASCs, Ab secreting cells; BAL, Bronchoalveolar lavage; CLN, cervical lymph node; CNS, central nervous system; dpi, days post infection; EID, egg infective dose; HA, hemagglutinin; HI, Hemagglutination inhibition; i.n., intranasal; i.m., intramuscular; nAbs, neutralizing antibodies; SIgA, secretory IgA; sHA1, swine HA1; s.l., sublingual; SN, Serum neutralization

responsible for vaccine efficacy (Hebert *et al*., 1997; Skehel and Wiley, 2000).

Mucosal immune responses serve as the first line of defense since mucosal surfaces are the main entrance of pathogens including influenza virus (Brandtzaeg and Pabst, 2004; Holmgren and Czerkinsky, 2005). Intranasal (i.n.) immunization induces secretory IgA (SIgA) in the airways as well as systemic immune responses (Yuki and Kiyono, 2009). However, it raised concerns about neurological side effects by which the vaccine is potentially directed to central nervous system (CNS) (Fujihashi *et al*., 2002; Armstrong *et al*., 2005). Sublingual (s.l.) route is commonly used for treatment against allergies (Akdis, 2008). Our previous studies demonstrated that s.l. immunization with ovalbumin or inactivated virus induces mucosal immune responses including induction of antigen (Ag)-specific SIgA, as well as systemic immune responses (Cuburu *et al*., 2007; Song *et al*., 2008b). Importantly, unlike i.n. route, s.l. administration did not redirect the vaccines to CNS (Cuburu *et al*., 2007; Song *et al*., 2008b).

In present study, we explored a novel approach of administering bacterially-expressed HA1 protein derived from influenza A/CA/04/2009 (H1N1) virus via sublingual route to induce protective immunity against 2009 pandemic influenza virus.

Materials and Methods

Construction of plasmid encoding HA1 (sHA1) protein of 2009 pandemic H1N1

The cDNA of HA from the influenza A/CA/04/09 (H1N1) was synthesized by GenScript Co. (USA). The gene (sHA1) corresponding to HA1 (residues 18-344) from 2009 H1N1 was amplified from the cDNA by polymerase chain reaction (PCR) with a forward primer (5′-GGGCCCGAATTCCCG ACACATTATGTATAGGT-3′) and reverse primer (5′-CC GTCTATTCAATCTAGACTCGAGGGGCCC-3′). The PCR product was digested with *Eco*RI and *Xho*I, fused with His-tag at the C terminus and inserted into the bacterial expression vector pET21d (+) (Novagen, USA). The resulted plasmid pET21d-sHA1 encodes expected size of HA1 of influenza A/CA/04/09 (H1N1) virus (Fig. 1A).

Expression and purification of the sHA1 protein

The sHA1 protein was expressed in *E. coli* BL21 (DE3) strain (Novagen) transformed with the pET21d-sHA1 plasmid. The cell pellets were suspended in a lysis buffer (20 mM Tris, 50 mM NaCl, pH 7.9) and disrupted by sonication on ice. The soluble and insoluble fractions were separated by centrifugation and the insoluble fraction was dissolved in binding buffer (20 mM Tris, 0.5 M NaCl, and 6 M urea, pH 7.9). After centrifugation at 20,000 rpm for 40 min, the supernatant was applied to Talon metal affinity column (Clontech, USA). The column was pre-washed with binding buffer, followed by wash buffer (20 mM Tris, 0.5 M NaCl, and 20 mM imidazole, pH 7.9) without urea. The protein was eluted with elution buffer (20 mM Tris, 0.5 M NaCl, and 0.3 M imidazole, pH 7.9) and then was dialyzed against 1× PBS. The purified protein was treated with 1% Triton X-114 to remove endotoxin and incubated with rocking for 30 min at 4°C, followed by incubation in a 37°C water bath for 20 min. The phase containing endotoxin was separated by centrifugation at 13,000 rpm for 5 min. This cycle was repeated five times. The protein was incubated with SM-2 beads (Bio-Rad, USA) for 2 h at 4°C to remove residual Triton X-114. Endotoxin level was less than 5 EU/mg as determined by the limulus amebocyte lysate (LAL) assay kit according to the instructions (Lonza, Switzerland). The purified protein preparation was examined on 12.5% SDS-PAGE and the protein concentration was determined by Bradford protein assay kit (Bio-Rad). The purified protein was stored at -80°C.

Immunization

Six-weeks-old female BALB/c mice were purchased from Orient Co. (Korea). All mice were maintained under specific pathogen-free conditions and the experiments involving animals were approved by Institutional Animal Care and Use Committees (IACUC) at Chungbuk University (BLS2011-0003) and International Vaccine Institute (2010-015). Mice (n=6) were immunized s.l. with 20 μg of sHA1 protein alone, or with 2 μg of cholera toxin (CT) (LIST BIOLOGICAL LABS INC., USA). For comparison, other groups of the mice were immunized i.m. with 20 μg of sHA1 protein in presence of 10 μg of Alum (Thermo scientific, USA) on days 0, 14 and 28.

Sample collection

Sera and mucosal samples were collected on day 7 after the last immunization. Blood was collected from the retro-orbital plexus and the sera were obtained by centrifugation. Prior to the collection of saliva, mice were injected intraperitoneally (i.p.) with pilocarpine (100 μg/animal) (Sigma, USA). Bronchoalveolar lavage (BAL) was collected by repeated flushing and aspiring with 500 μl of PBS from the lungs. Nasal washes were collected by flushing with 50 μl of PBS two times through the nasal cavity. The samples were stored at -80°C until used.

Measurement of specific Abs

Ab titers in sera and mucosal secretions were measured by

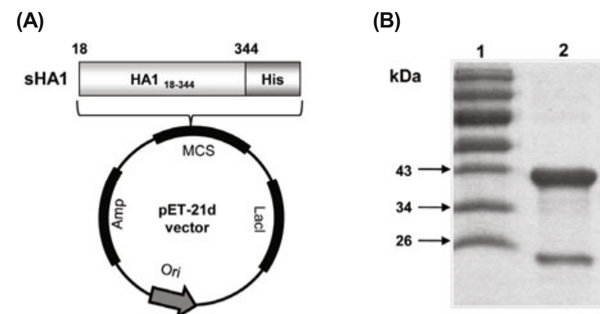

Fig. 1. Construction and purification of the sHA1 protein. (A) The gene corresponding to HA1 (residues 18-344) from influenza A/CA/04/2009 (H1N1) virus was cloned into the pET-21d bacterial expression vector. (B) The sHA1 protein expressed in *E. coli* was purified by His-tag affinity chromatography and confirmed by SDS-PAGE. Lanes: 1, protein molecular weight marker; 2, the purified HA1 protein.

enzyme-linked immunosorbent assay (ELISA). The 96-well ELISA plates (Nunc, Denmark) were pre-coated with sHA1 protein (2 μg/ml) in 50 mM Sodium bicarbonate buffer (pH 9.6). The plates were washed with PBST and incubated with blocking buffer (5% Skim milk in PBS) for 1 h. Serial 2- or 3-fold diluted samples in blocking buffer were added to each well and incubated for 1 h at 37°C. After washes with PBST, HRP-conjugated goat anti-mouse IgA, IgG, IgG1 or IgG2a (Southern Biotechnology Associates, USA) was added to each well, and incubated at RT for 1 h. Peroxidase substrate tetramethylbenzidine (TMB) (Millipore, USA) was added to each well. The reaction was stopped by adding 0.5 N HCl. The absorbance at wavelength 450 nm was recorded by an ELISA reader (Molecular Devices, USA). The endpoint titer was determined by OD cut-off values of 0.2.

Hemagglutination inhibition (HI) and Serum neutralization (SN) assay

The HI assay was performed as described previously (Song *et al.*, 2009). Briefly, 50 μl of 1× PBS was dispensed in a 96-well microtiter plate. Sera treated with receptor destroying enzyme (RDE) were serial 2-fold diluted and added to each well. The diluted sera were incubated with 4 HA units of H1N1 virus at RT for 30 min, followed by the addition of 0.5% turkey red blood cells (RBCs). HI titers were defined as the highest dilution that inhibited agglutination. SN assay was performed as described previously (Kim *et al.*, 2008). Madin-Darby canine kidney (MDCK) cells were seeded at 1.5×10^4 cells/well in a 96-well plate and incubated for 4 h at 37°C in CO_2 incubator. The sera were serially diluted 2-fold and 50 μl of diluents was mixed with 50 μl (100 $TCID_{50}$) of the A/CA/04/09 (H1N1) virus for 30 min. Each mixture was added to the 96-well plate and incubated for 24 h. The plate was fixed with 80% acetone and washed with 1× PBS. Anti-influenza A NP (Millipore) were added to each well and incubated for 1 h at 37°C, followed by the addition of 1:3,000 diluted horseradish peroxidase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates). After incubation for 1 h at room temperature, o-Phenylenediamine dyhydrochloride (OPD) (Sigma) was added to each well and then the absorbance at wavelength 450 nm was recorded by an ELISA reader. The endpoint titer was determined with the value less than X, where X is calculated as (average *A*450 of virus infected wells – average *A*450 of negative wells)/2 + average *A*450 of negative wells.

Virus titers in lung tissues

BALB/c mice were immunized three times as described above and challenged i.n. with 30 μl (3×10^3 $TCID_{50}$) of the A/CA/04/09 (H1N1) virus five weeks after the last vaccination. Mice were continuously observed for 12 days post infection (dpi). Lung tissue samples of the mice were collected at 5 dpi and homogenized in 1× PBS containing antibiotics. Tissue homogenates were centrifuged and supernatants were transferred to new tubes. All samples were serially diluted 10-fold and then inoculated into 11-day-old embryonated chicken eggs for virus titration as computed by the Reed and Muench method with results expressed as $\log_{10}$ 50% egg infective dose (EID) per ml of tissue collected (EID_{50}/ml) (Reed and Muench, 1938). All experiments were carried out in the biosafety level 3 (BSL-3+) facilities and conditions.

ELISPOT assay

HA-specific Ab secreting cells (ASCs) in spleen, cervical lymph node (CLN) and lung were enumerated by ELISPOT assay (Cuburu *et al.*, 2007). 96-well nitrocellulose microplates (Millipore) were coated with sHA1 protein (10 μg/ml). One million cells isolated from each organ were transferred to the plates at 2-fold dilutions, followed by addition of HRP conjugated goat anti-mouse IgG or IgA (Southern Biotechnology Associates). After incubation for 4 h, the plates were extensively washed with PBS, PBS-T, water, sequentially. Spots were developed by addition of AEC-H_2O_2 chromogenic substrate (Sigma-Aldrich) and counted by using an ELISPOT reader (Molecular devices).

Statistical analysis

All data were expressed as mean value+SD. The Student's t-test was used for comparisons between experimental groups. A value of $P<0.05$ was considered as significant.

Results

Expression and purification of the sHA1 protein from bacterial system

Since most antigenic sites within the influenza HA protein are located in HA1 domain (Shih *et al.*, 2007), we chose sHA1 (residues 18-344) domain of the HA from influenza A/CA/04/2009 (H1N1) virus. The gene was inserted into pET-21d (+) plasmid to express as a fusion of His-tag at the C terminus. The sHA1 protein was expressed in *E. coli* and purified using His-tag affinity chromatography (Fig. 1A). SDS-PAGE revealed expected recombinant protein at corresponding molecular weight, 39 kDa (Fig. 1B).

S.l. immunization with the sHA1 protein induced humoral immune responses

We first evaluated the Ab responses induced in both mucosal and systemic compartments upon s.l. and i.m. immunizations. BALB/c mice were immunized s.l. three times 2 weeks apart with 20 μg of sHA1 protein alone or sHA1 mixed with CT, or i.m. with 20 μg of sHA1 protein mixed with Alum. The sera were harvested on day 7 after the last immunization and sHA1-specific serum IgG, IgG1, and IgG2a Ab titers were determined by ELISA. As shown in Fig. 2A, CT-adjuvanted sHA1 protein induced significantly ($P<0.05$) higher titer of sHA1-specific IgG in the sera as compared to sHA1 protein alone. Of note, s.l immunization with sHA1 mixed with CT induced Ag-specific IgG titer in sera comparable to that induced by i.m. route. IgG1 was induced predominantly in the blood of mice immunized by either s.l. or i.m. route.

SIgA is known to play an important role in protection against influenza virus infection of respiratory tract (Renegar and Small Jr, 1991). We next determined levels of sHA1-specific SIgA in saliva, nasal wash and BAL using ELISA. As shown

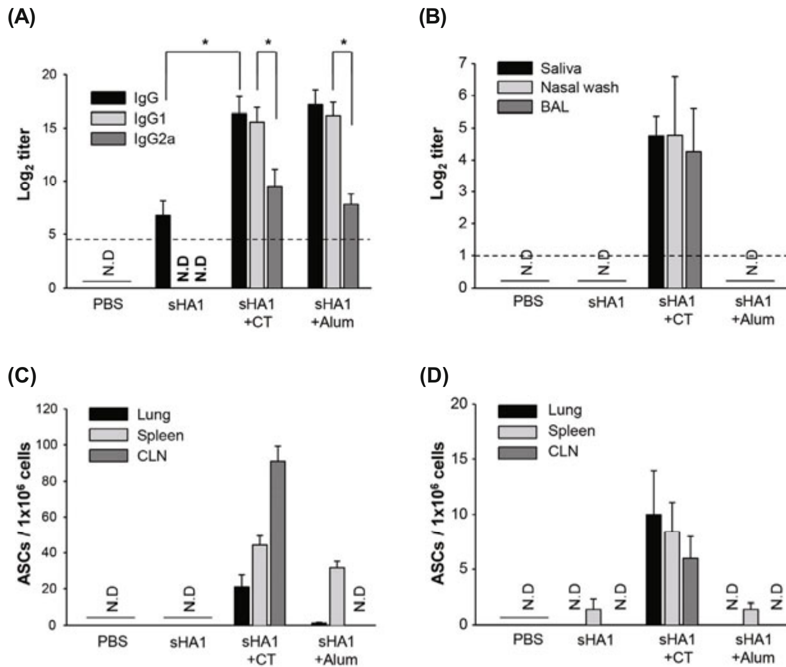

Fig. 2. **Ag-specific humoral responses in sHA1 protein-immunized mice.** BALB/c mice were immunized three times with 20 μg of sHA1 protein alone or mixed with 2 μg of CT by s.l. route, or with 20 μg sHA1 mixed with 10 μg of Alum by i.m. route. Control mice were immunized with PBS. Serum and mucosal secretions were collected on day 7 after the last immunization. (A) sHA1-specific serum IgG, IgG1, and IgG2a titers and (B) sHA1-specific saliva, nasal wash and BAL IgA titers were measured by ELISA. sHA1-specific IgG (C) and IgA (D) ASCs in spleen, lung and CLN were measured by ELISPOT assays on day 7 after the last immunization. The dashed line indicates detection limit. ND indicates not detected. The data are representative of three independent experiments and expressed as the Mean±SD for the group. Significant differences are expressed as $^{*}P<0.05$.

in Fig. 2B, significant levels of sHA1-specific SIgA were observed in saliva, nasal wash and BAL of mice immunized with CT-adjuvanted sHA1. Consistently, s.l. immunization with sHA1 protein in presence of CT induced higher numbers of HA-specific IgG and IgA ASCs in the lung, spleen and CLN as compared to s.l. immunization with sHA1 protein alone or i.m. immunization with sHA1 protein mixed with alum (Figs. 2C and 2D). The results indicate that s.l. immunization of sHA1 protein with CT induces significant Ab responses in both systemic and mucosal compartments.

S.l. immunization with sHA1 protein induced nAb against pandemic H1N1 influenza virus.

Since protection against influenza virus infection is mediated by nAbs that bind to HA (Dormitzer *et al*., 2011), we next examined whether sHA1 protein induces nAb upon s.l. immunization. Indeed, significant HI and SN titers (1,024 and 512, respectively) against 2009 pandemic H1N1 virus strain were observed in the sera collected on day 7 after the last immunization (Figs. 3A and 3B). The titers of serum nAb induced by s.l. immunization with CT-adjuvanted sHA1 protein were comparable to those induced by i.m. immunization with Alum-adjuvanted sHA1 protein. These results demonstrate that s.l. immunization of the non-glycosylated sHA1 protein with CT induces readily protective nAb.

S.l. immunization with sHA1 protein vaccine provided protection against infection with 2009 pandemic H1N1 influenza virus.

We further tested whether s.l. immunization with sHA1 protein induces protection against infection with pandemic H1N1 influenza virus. The immunized mice were challenged i.n. with influenza A/CA/04/2009 (H1N1) virus. Five days after the challenge, viral titers in the lungs were determined by EID_{50} assay. As expected, virus titers in the lungs of mice immunized with adjuvanted sHA1 protein were 10,000-fold lower than those in the lungs of control groups that received PBS or sHA1 alone (Fig. 4A). The latter groups lost 15% of their initial body weight after the challenge while mice immunized s.l. with CT adjuvanted sHA1 protein lost 10% of their initial body weight and recovered readily on day 6 after the challenge (Fig. 4B). It was noting that mice vaccinated

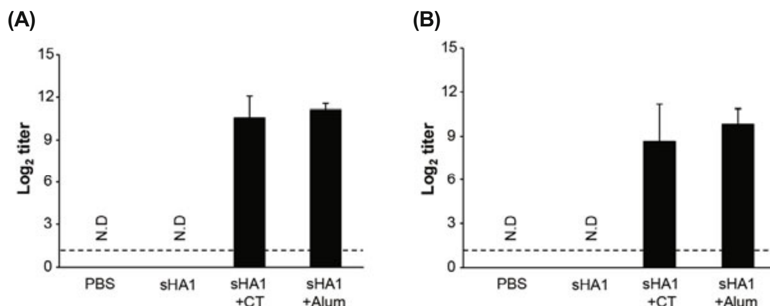

Fig. 3. **Neutralizing activity of sera against 2009 pandemic influenza A virus (H1N1).** Serum samples were collected on day 7 after the last immunization. (A) The sera were assayed for HI and the serial two-fold diluted sera were incubated with 4 HA units of A/CA/04/09 (H1N1) virus, followed by addition of RBCs. Data represents the highest dilution of serum exhibiting complete inhibition of hemagglutination. (B) The sera were used for SN assay and the serial two-fold diluted sera were incubated with of A/CA/04/09 (H1N1) virus (100 $TCID_{50}$) in MDCK cells. The Data represents the highest dilution of serum inhibiting of virus infection in MDCK cells. The dashed line indicates detection limit. ND indicates not detected. The data are representative of three independent experiments and expressed as the Mean± SD for the group.

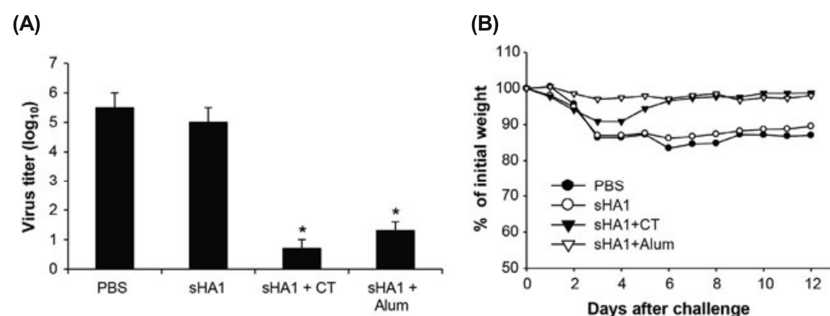

Fig. 4. Protection against the 2009 pandemic influenza A virus (H1N1). Mice were challenged with 3×10^3 $TCID_{50}$ of the A/CA/04/09 (H1N1) virus five weeks after the last immunization. (A) Viral titers in the lung tissue on day 5 after the challenge were determined in embryonated chicken eggs. Lung viral titers are expressed as log_{10} EID_{50}. Significant differences compared with PBS were expressed as $^*P<0.05$. (B) Body weight was monitored daily after the viral challenge. The data are expressed as the Mean±SD for the group.

i.m. with Alum adjuvanted sHA1 protein did not show significant weight loss. The results show that s.l. immunization with sHA1 protein in the presence of CT provides protection against infection with pandemic H1N1 influenza virus in mice.

Discussion

In the present study, we demonstrated for the first time, at the best of our knowledge, that s.l. vaccination of the non-glycosylated recombinant sHA1 protein with CT as the mucosal adjuvant provided protection against influenza virus infection in mice. This approach was examined using 2009 pandemic influenza virus as a model. Current novel vaccination strategy induced protective Ab responses in systemic and mucosal compartments. Influenza HA glycoprotein is a major viral antigenic component against which nAbs are produced. These HA-specific nAbs play an important role in protection against infection with influenza virus (Ahmed *et al*., 2007). Since the antigenic sites of HA are conformational epitopes (Stevens *et al*., 2004), glycosylation has been considered as a key factor for proper folding (Hebert *et al*., 1997) and binding to host cells (Skehel and Wiley, 2000). On the other hand, there are evidences suggesting that a non-glycosylated subunit protein was highly immunogenic and induced protective immunity (Song *et al*., 2008a; Aguilar-Yanez *et al*., 2010). In the line of evidences to support this, our study clearly showed that s.l. immunization with non-glycosylated sHA1 protein in the presence of CT as mucosal adjuvant induced nAbs.

Until now, embryonic chicken egg-based vaccines have been produced and widely used against influenza infection. However, during a pandemic egg-based vaccine is vulnerable to be scaled up in a short period of time in order to meet the demand because of the limited supplies of high-quality eggs (Verhoeyen *et al*., 1980). Bacteria expression system is cost-effective and produces mass amounts of recombinant protein-based vaccines rapidly (Aguilar-Yanez *et al*., 2010). Thus this approach offers an alternative and/or additional option for producing a large quantity of influenza vaccine, especially at the early stage of pandemic.

Mucosal immunity is important for the first line of defense because almost all pathogens enter their hosts through a mucosal site (Holmgren and Czerkinsky, 2005). Particularly, mucosal SIgA plays a critical role in protecting animals against influenza virus infection (Liew *et al*., 1984; Renegar and Small Jr, 1991). Nevertheless, the traditional influenza vaccines are designed for systemic administrations such as i.m. and subcutaneous (s.c.) that may have limited effect on the induction of mucosal immune responses. A number of recent studies demonstrated that i.n. immunization of recombinant HA subunit vaccine with an adjuvant provided protection against influenza virus infection (Asahi-Ozaki *et al*., 2006; Meng *et al*., 2011). However, i.n. route remains a safety issue associated with directing the viral vaccine to CNS causing neurological side effects (Fujihashi *et al*., 2002; Armstrong *et al*., 2005). Previously, we showed that s.l. administration did not redirect antigens to the CNS (Cuburu *et al*., 2007; Song *et al*., 2008b). In the current study, we showed that s.l. immunization of sHA1 protein with CT induced not only IgG in the blood, but also SIgA in saliva, BAL and nasal wash. Furthermore, the s.l. immunization provided a protection where the vaccinated mice showed less body weight loss and significantly lower virus titers in lungs as compared to unimmunized mice. It is to note that during a pandemic, there is a concern about the availability of medical specialists to administer vaccines in developing countries. For this, s.l. immunization could be an attractive method for influenza vaccination, because it does not require special delivery devices (Agostinis *et al*., 2005; Cuburu *et al*., 2007) and medical training.

In conclusion, s.l. immunization with recombinant sHA1 in the presence of a mucosal adjuvant offers a novel, safe, cost-effective and convenient vaccination strategy to combat influenza outbreaks.

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