Microbiology

Vaccination with Killed but Metabolically Active *E. coli* Over-expressing Hemagglutinin Elicits Neutralizing Antibodies to H1N1 Swine Origin Influenza A Virus

Pei-Feng Liu[1], Yanhan Wang[1], Yu-Tsueng Liu[2,3], and Chun-Ming Huang[1,3,*]

[1] Department of Dermatology, University of California, San Diego, San Diego, USA. [2] Division of Infectious Diseases, Department of Medicine, University of California, San Diego, USA. [3] Moores Cancer Center, University of California, San Diego, San Diego, USA

There is a need for a fast and simple method for vaccine production to keep up with the pace of a rapidly spreading virus in the early phases of the influenza pandemic. The use of whole viruses produced in chicken eggs or recombinant antigens purified from various expression systems has presented considerable challenges, especially with lengthy processing times. Here, we use the killed but metabolically active (KBMA) *Escherichia coli* (*E. coli*) to harbor the hemagglutinin (HA) of swine origin influenza A (H1N1) virus (S-OIV) San Diego/01/09 (SD/H1N1-S-OIV). Intranasal vaccination of mice with KBMA *E. coli* SD/H1N1-S-OIV HA without adding exogenous adjuvants provoked detectable neutralizing antibodies against the virus-induced hemagglutination within three weeks. Boosting vaccination enhanced the titers of neutralizing antibodies, which can decrease viral infectivity in Madin-Darby canine kidney (MDCK) cells. The antibodies were found to specifically neutralize the SD/H1N1-S-OIV-, but not seasonal influenza viruses (H1N1 and H3N2), -induced hemagglutination. The use of KBMA *E. coli* as an egg-free system to produce anti-influenza vaccines makes unnecessary the rigorous purification of an antigen prior to immunization, providing an alternative modality to combat influenza virus in future outbreaks.

H1N1 swine origin influenza A virus | Hemagglutinin | KBMA | microbe based-vaccine |Intranasal vaccine

Introduction

Seasonal influenza causes thousands of deaths annually. In the United States (US) alone, more than 50,000 patients die yearly due to influenza-like illness and its consequences (1). In mid-April 2009, a new swine origin influenza A (H1N1) virus (S-OIV) emerged in US (2) and Mexico (3,4). The virus quickly spread worldwide to many countries through human-to-human transmission. In the period between April-December 2009, millions of people were infected. By the end of the year 2009, WHO had declared that 12,000 persons had died due to influenza A/H1N1; half of them in North America (Mexico, Canada and US) (3). Current vaccines, which are strain-specific, pose logistical challenges in terms of rapid production and widespread availability during a pandemic caused by a new strain of influenza virus. Thus, there is an urgent need to develop a rapid modality to manufacture large quantities of influenza vaccines in order to cope with the requests of a pandemic (2).

The traditional egg-based approach to producing influenza vaccines does not provide sufficient capacity and adequate speed to satisfy global needs to combat newly emerging strains, seasonal or potentially pandemic (5), because it needs to select virus variants (6), separates and inactivates viral particles, and to purify the primary vaccine antigen (2). Eggs must be ordered up to a year in advance, necessitating careful planning every year to ensure a sufficient supply of fertile eggs (7). Moreover, some egg-allergic individuals may react to the flu vaccines containing egg (8). Alternatively, several strategies include viral culture in mammalian cells (9-11) or insect cells (12,13) have been proposed to produce pandemic and seasonal influenza vaccines (9,14). However, the primary disadvantages are the low levels of expression of proteins in mammalian or insect cells as well as the added unknown risks of antigens produced from mammalian cells containing other potential sources of contamination (15).

In contrast, the expression of antigenic proteins in bacterial culture (5,16,17) is one of simple and fast strategies for generating large quantities of influenza vaccines and reducing whole virus vaccine-induced complications such as pyogenic reaction and Guillain-Barre syndrome (18). The use of a killed but metabolically active (KBMA) microbe for vaccination is a new paradigm for eliciting protective immunity (19,20) KBMA bacteria are rendered noninfectious by the combined use of psoralen and long-wave ultraviolet (UV) light and abrogation of nucleotide excision repair through deletion of the bacterial *uvrAB* genes. Vaccination with KBMA *Bacillus anthracis* (*B. anthracis*) fully protected mice and rabbits against challenge with lethal doses of bacterial spores (21). Here, we use the UV-inactivated *Escherichia coli* (*E. coli)* over-expressing hemagglutinin (HA) of H1N1 S-OIV San Diego/01/09 (SD/H1N1-S-OIV) as a KBMA microbe-based influenza vaccine (KBMA *E. coli* HA SD/H1N1-S-OIV). It has been reported that animals can be protected against

Abbreviations: ACP, acid phosphatase; *B. anthracis, Bacillus anthracis*; BSA, bovine serum albumin; CAMP, Christie-Atkins-Munch-Peterson; cDNA, complementary DNA; CFU, colony forming unit; DMEM, Dulbecco's modified Eagle's medium; *E. coli*, *Escherichia coli*; GFP, green fluorescent protein; HA, hemagglutinin; HAU, hemagglutinating unit; HI, hemagglutination inhibition*;* HIA, hemagglutination inhibition assay; ICR, Institute of Cancer Research; IPTG, isopropyl-β-D-thiogalactoside; KBMA, killed but metabolically active; LB, Luria-Bertani; MDCK, Madin-Darby canine kidney; OD, optical density; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; *P. acnes*, *Propionibacterium acnes*; PVDF, polyvinylidene fluoride; SA, sialic acid; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SE, standard error; S-OIV, swine origin influenza A virus; SD/H1N1-S-OIV, San Diego/01/09 S-OIV; US, United States; UV, ultraviolet

Conflict of interest: No conflicts declared.

* Corresponding Author: Professor, Chun-Ming Huang, Department of Dermatology, University of California, San Diego. 3525 John Hopkins Court, Rm276, San Diego, CA, 92121, USA.
Tel: 858-822-4627; Fax: 858-642-1435.
E-mail: chunming@ucsd.edu

microbe-based influenza vaccine (KBMA *E. coli* HA SD/H1N1-S-OIV). It has been reported that animals can be protected against lethal infection by *Clostridium tetani* cells and *B. anthracis* spores following topical application of intact particles of KBMA (gamma-irradiated) *E. coli* over-expressing tetanus and anthrax antigens, respectively (22). In our laboratory, KBMA *E. coli* created by UV inactivation has been extensively used as a vectorsystem to harbor various antigens (30-32). For example, the vaccination of KBMA *E. coli* over-expressing Christie-Atkins-Munch-Peterson (CAMP) factor of *Propionibacterium acnes* (*P. acnes*) (23) or hemolysins of *Staphylococcus aureus* (24) abrogated the bacteria-induced skin infections in mice. The vaccination of KBMA *E. coli* over-expressing a surface FomA protein of *Fusobacterium nucleatum* efficiently reduced the oral abscesses in mice (25) When the KBMA *E. coli* over-expressing antigens are used as vaccines, they contain natural immunomodulators or adjuvants to boost immunity against live pathogens (19,20) eliminating the time-consuming steps required for adjuvant choices and antigen purification. *E. coli* has been recognized as a member of human commensals (26) though administration of *E. coli* containing enterotoxin may induce adverse reactions. A Phase I study had shown that intranasal vaccines using purified HA along with *E. coli* enterotoxin were well tolerated in humans (27). Furthermore, the use of heat-killed *E. coli* K12 for oral administration has been proven safe in clinical trials (28,29).

In this study, we document the production of HA of SD/H1N1-S-OIV) in KBMA *E. coli* and demonstrate that intranasal vaccination with KBMA *E. coli* HA SD/H1N1-S-OIV in mice provokes potent neutralizing antibodies which elicit a specific inhibitory effect on SD/H1N1-S-OIV-, neither seasonal influenza H1N1- nor H3N2-, induced hemagglutination. Intranasal vaccination with KBMA *E. coli* HA SD/H1N1-S-OIV provides a new platform for rapid large scale production of influenza vaccines in the face of an influenza pandemic threat.

Materials and Methods

Molecular cloning, expression, and purification of recombinant HA of SD/H1N1-S-OIV

The complementary DNA (cDNA) of SD/H1N1-S-OIV was used as a template (30) to amplify a polymerase chain reaction (PCR) product encoding SD/H1N1-S-OIV HA (Accession number: ACP41105). The forward PCR primer (5'-AATAGTCGACATGAAGGCAATACTAGTAGTTCTGCTAT AT-3') and the reverse PCR primer (5'-GATACAGCGGCCG CTTAAATACATATTCTACACTGTAGAGACCCA-3') were designed for a PCR reaction. The amplified DNA products were inserted into the In-Fusion Ready pEcoli-Nterm 6×HN vector (Clontech Laboratories, Mountain View, CA) and transformed into competent cells [(*E. coli*), BL21 (DE3), Invitrogen, Carlsbad, CA]. As a control, a pEcoli-Nterm-green fluorescent protein (GFP) plasmid (Clontech Laboratories Inc.) was transformed. After isopropyl β-D-thiogalactopyranoside (IPTG) (1 mM) induction and centrifugation at 3,000 × *g* at 4°C for 5 min, bacterial pellets were re-suspended with sodium dodecyl sulfate (SDS) loading buffer [125 mM Tris-HCl buffer, pH 6.8, containing 4% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.002% (w/v) bromophenol blue] and then boiled for 5 min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 10%) and subsequent gel staining with coomassie blue were used for detection of protein expression. Expression of induced HA of H1N1-S-OIV in *E. coli*, BL21 (DE3) was quantified with Software Gel-Pro Analyzer 4.0 (Fig. 1). The fusion protein was purified from IPTG-induced bacteria inclusion bodies collected by centrifugation at 3,000 × *g* at 4°C for 5 min. Subsequently, the inclusion bodies were refolded as recommended by the manufacture (Amersham Biosciences, Piscataway, NJ). To further confirm the protein expression, the lysates of *E. coli*, BL21(DE3) harboring SD/H1N1-S-OIV HA, GFP, or *P. acnes* CAMP factor were subjected to 10 % SDS-PAGE, immunoblotted on polyvinylidene fluoride (PVDF) membranes and incubated with sera from mice immunized with KBMA *E. coli* HA SD/H1N1-S-OIV or a commercial antibody to swine origin influenza A (H1N1) HA (ProSci Incorporated, Poway, CA, USA).

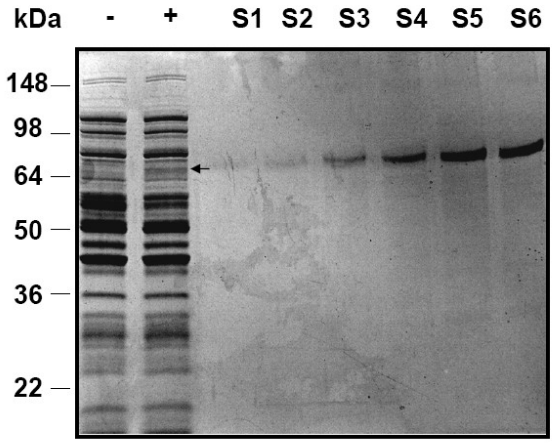

Figure 1. Estimation of the expression level of HA of H1N1-S-OIV in *E. coli*, BL21 (DE3) (1 × 10^8 CFU). The HA of SD/H1N1-S-OIV was expressed in *E. coli* in the absence (-) or presence (+) of 1 mM IPTG. After IPTG induction, the expression of HA with approximately 64 kDa was indicated (arrow) on a 10% SDS-PAGE. The intensities of protein bands corresponding with HA and different concentrations of BSA (S1-S6) were quantified by Gel-Pro Analyzer 4.0 software. The expression level of HA was estimated by the same intensity of HA as a concentration of BSA.

Virus growth

A clinical isolate of SD/H1N1-S-OIV and two seasonal influenza A/Brisbane/59/2007 (H1N1) virus (ATCCH1N1) and an A/Brisbane/10/2007 (H3N2)-like virus (ATCCH3N2) were grown in virus growth medium containing Dulbecco's modified Eagle's medium (DMEM) with glutamine, 0.2% bovine serum albumin (BSA), 25 mM Hepes and 2 μg/ml TPCK-trypsin (Sigma Chemical Co., St. Louis, Mo.) and propagated in Madin-Darby canine kidney (MDCK) cells as described in our previous publication (31).

Intranasal immunization with KBMA E. coli HA SD/H1N1-S-OIV

To create KBMA *E. coli*, the *E. coli* BL21(DE3) over-expressing HA of SD/H1N1-S-OIV or GFP was inactivated by UV irradiation at total energy of 7000 J/m^2 by a Spectrolinker (Spectronics, Westbury, NY) (23,25,32). The viability of UV-irradiated *E. coli* BL21(DE3) cell was determined by observing the growth of bacterial colonies on Luria-Bertani (LB) agar plates (Fig. 2). For immunization, female Institute of Cancer Research (ICR) mice approximately 3-6 week-old (Harlan, Indianapolis, IN) were intranasally immunized with 1× 10^8 colony forming unit (CFU) of UV-irradiated *E. coli* BL21(DE3) H1N1-S-OIV HA containing over-expressed HA protein or GFP for three weeks. The second and third inoculations were administered in the same manner as the first immunization. Purified H1N1-S-OIV HA (10 μg) or GFP (10 μg) was subjected to 10 % SDS-PAGE,

immunoblotted on PVDF membranes and incubated with serum obtained from mice immunized with KBMA *E. coli* over-expressing HA or GFP for 3 weeks (1: 500 dilution).

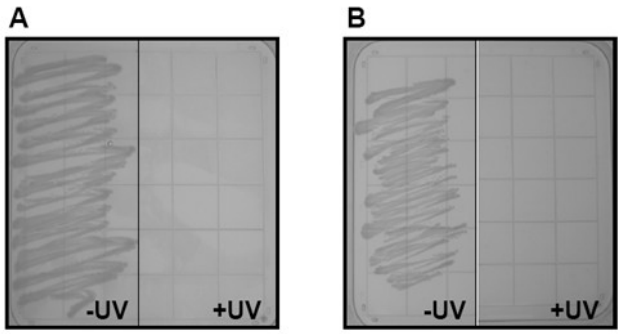

Figure 2. Bacterial inactivation by UV irradiation. The 1×10^8 CFU of (A) *E. coli* BL21(DE3) GFP or (B) *E. coli* BL21(DE3) SD/H1N1-S-OIV HA with (+ UV) or without (-UV) irradiation was cultured on LB agar plates, respectively. Bacterial inactivation via UV irradiation (7,000 J/m^2) was illustrated by the inability to grow on agar plates.

Serum hemagglutination inhibition (HI) antibody titer measurement

Serum HI antibody titers in mice immunized with KBMA *E. coli* over-expressing HA or GFP for 3-6 weeks were analyzed by HI assay (HIA). HIA was performed in v-bottom 96-well microtiter plates (Costar, Cambridge, MA) at room temperature for 40 min. Hemagglutination titers were determined by titration of 2-16 hemagglutinating unit (HAU) (50 µl) of SD/H1N1-S-OIV, 16 HAU of seasonal ATCCH1N1 (50 µl) or 2 HAU (50 µl) of seasonal ATCCH3N2 in phosphate-buffered saline (PBS) followed by addition of serum (25 µl) obtained from immunized mice. Afterward, equivalent volume of guinea pig erythrocytes (25 µl; Lonza, Walkersville, Inc. MD) was added and the ability of serum to inhibit the virus-induced hemagglutination was assessed. All sera were heat (56°C for 5 min)-inactivated to destroy complement prior to performing neutralizing assays.

Selectivity of antibodies to HA of swine origin influenza A (H1N1)

Recombinant HA (10 µg) of SD/H1N1-S-OIV or seasonal ATCCH1N1 (Abcam Inc. Cambridge, MA) was loaded into a 10 % SDS-PAGE, immunoblotted on PVDF membranes and incubated with a commercial antibody to swine origin influenza A (H1N1) HA (1:500 dilution). Peroxidase activity was visualized with a western lighting chemiluminescence kit (PerkinElmer, Boston, MA).

Viability of MDCK cells infected with SD/H1N1-S-OIV

MDCK cells were grown in minimum essential medium containing 10% fetal bovine serum, 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate in a 96-well plate until confluence. Cells were then infected with a 1:100,000 dilution of SD/H1N1-S-OIV (16 HAU) for 2 days in the presence serum collected from mice six weeks after immunization with KBMA *E. coli* over-expressing HA or GFP. After that, cell viability was determined by an acid phosphatase (ACP) assay (33). Briefly, cells were washed with PBS three times and incubated with 100 µl of 10 mM *p*-nitrophenyl phosphate in an ACP assay buffer [1M sodium acetate buffer, pH5.5, containing 0.1% (w/v) Triton X-100] at 37°C for 1 h. Ten µl of 1 N NaOH was then added to stop the reaction before optical density (OD) at 405 nm was measured. The death of SD/H1N1-S-OIV-infected cells was calculated as the percentage of cell death caused by Triton X-100 (0.1%, v/v).

Statistical analyses

Data are presented as mean ± standard error (SE). Student *t*-test was used to assess the significance of independent experiments. The criterion (**p<0.005, ***p<0.0005) was used to determine statistical significance.

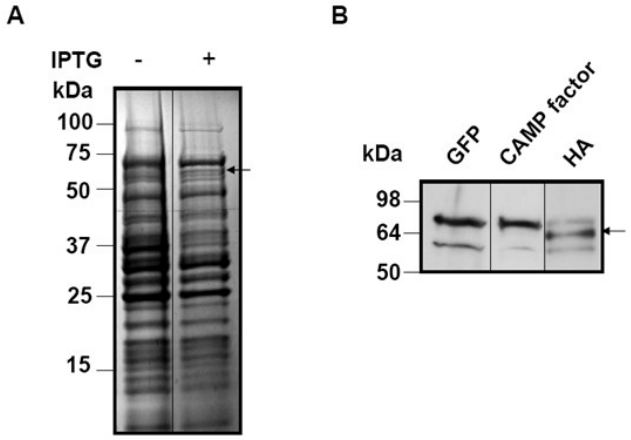

Figure 3. Expression and identification of recombinant HA of SD/H1N1-S-OIV. A pEcoli-6×HN-GFPuv vector was inserted with a full PCR amplified encoding SD/H1N1-S-OIV HA (Accession number: ACP41105.1). (A) Expression of HA of SD/H1N1-S-OIV in *E. coli* with (+) and without (-) IPTG induction. A protein band at approximately 64 kDa corresponding to HA (arrow) on a 10% SDS-PAGE was indicated. (B) The laystes of *E. coli* harboring GFP (GFP), *P. acnes* CAMP factor (CMAP factor) and HA of H1N1-S-OIV (HA) were subjected to 10 % SDS-PAGE, PVDF membranes and incubated with a commercial antibody to swine origin influenza A (H1N1) HA.

Results

Expression of SD/H1N1-S-OIV HA in E. coli

HA, the principal antigen on the surface of influenza virus, is responsible for viral binding to host receptors, enabling entry into the host cell through endocytosis and subsequent membrane fusion. As such, the HA is currently used as a common target for development of influenza virus vaccines (34). To express the HA of SD/H1N1-S-OIV, the PCR products of gene (ACP41105) encoding SD/H1N1-S-OIV HA were inserted into a pEcoli-6×HN plasmid and transformed into *E. coli* BL21(DE3). After IPTG induction, the over-expressed HA-6×HN fusion protein at approximately 64 kDa molecular weight was detected by SDS-PAGE with coomassie blue staining (Figs. 1 and 3A). The induced expression of SD/H1N1-S-OIV HA in *E. coli* was quantified with Software Gel-Pro Analyzer 4.0. As shown in Fig. 1, approximately 1.4 µg of SD/H1N1-S-OIV HA was expressed in 1×10^8 CFU of *E. coli* BL21(DE3) cells. To further validate the HA expression, the lysates *E. coli*, BL21(DE3) harboring SD/H1N1-S-OIV HA were subjected to 10 % SDS-PAGE and incubated with a commercial antibody to swine origin influenza A (H1N1) HA for Western blot analysis. The lysates *E. coli*, BL21(DE3) harboring GFP or *P. acnes* CAMP factor serve as controls. Antibody to swine origin influenza A (H1N1) HA recognizes a band (approximately 64 kDa) which is detected exclusively in the lysates of *E. coli* BL21(DE3) harboring SD/H1N1-S-OIV HA, but not GFP or *P. acnes* CAMP (Fig. 3B), verifying the expression of recombinant HA of SD/H1N1-S-OIV in *E. coli*.

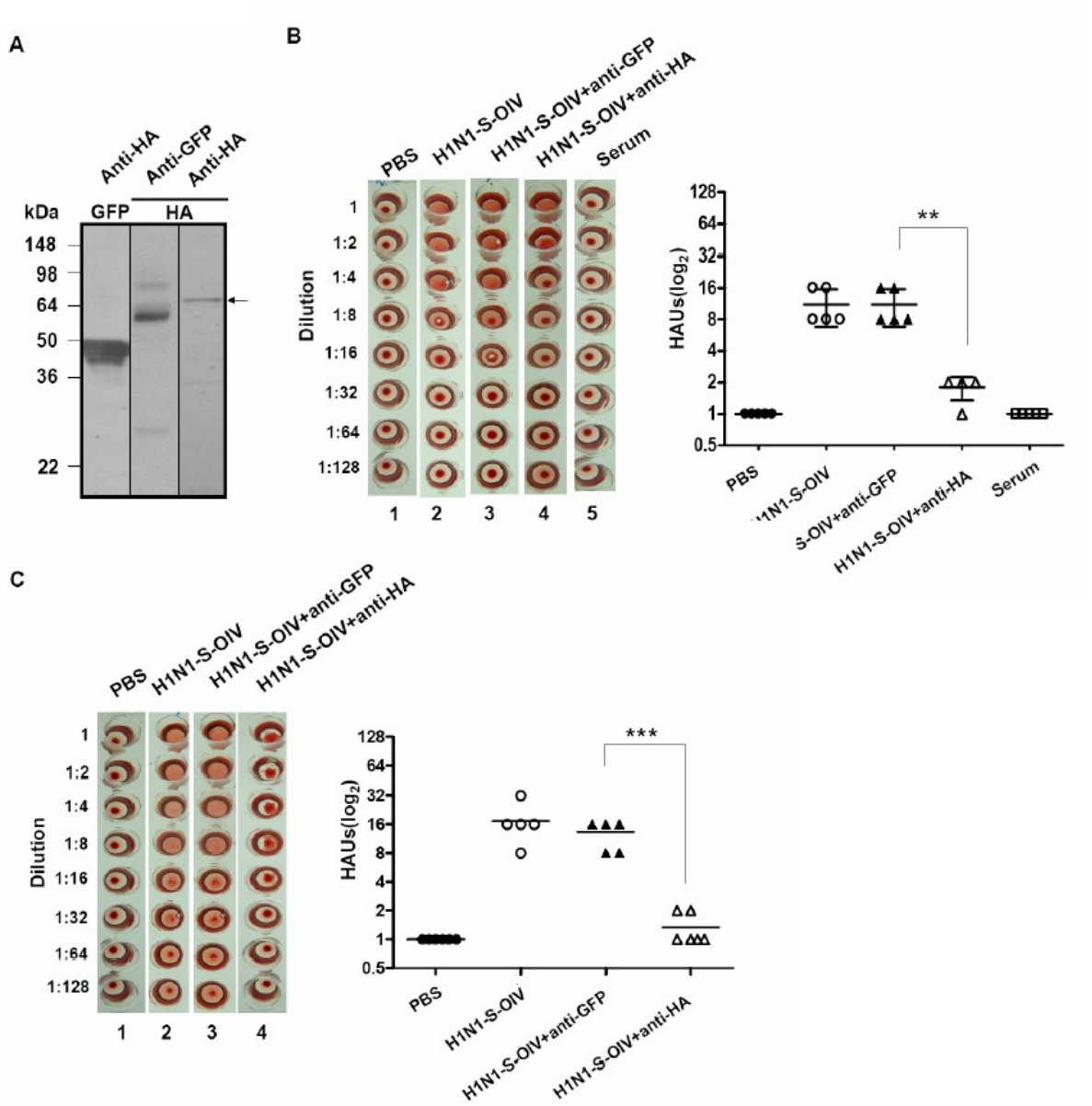

Figure 4. Inhibition of SD/H1N1-S-OIV-induced erythrocyte hemagglutination using neutralizing antibodies to SD/H1N1-*S*-OIV HA. (A) Western blot analysis demonstrated the recombinant HA (25 μg; arrow) of SD/H1N1-S-OIV was immunoreactive to mouse serum (1: 500 dilution) collected from three weeks after immunization with KBMA *E. coli* HA SD/H1N1-S-OIV (anti-HA), but not or KBMA *E. coli* GFP (anti-GFP). Recombinant GFP (25 μg; GFP) was reacted with anti-HA as a negative control. Neutralization of SD/H1N1-S-OIV-induced hemagglutination by anti-HA collected from mice three weeks (B) or six weeks (C) after immunization was illustrated. HIA was performed by incubating erythrocytes with PBS (lane 1), PBS plus twofold serial dilution (1-128) of SD/H1N1-S-OIV (8 HAU) (lane 2), SD/H1N1-S-OIV plus anti-GFP (lane 3) or SD/H1N1-S-OIV plus anti-HA (lane 4). Erythrocytes incubated with PBS plus naïve mouse serum in the absence of SD-H1N1-S-OIV (lane 5; Serum) served as a control. The inhibition of hemagglutination activity was expressed as log2 of HAUs and calculated from six independent experiments. Data are presented as mean ± SE. Student *t*-test was used to assess the significance of independent experiments. The criterion (**p<0.005, ***p<0.0005) was used to determine statistical significance.

Inhibition of SD/H1N1-S-OIV-induced hemagglutination by neutralizing antibody to HA

To assess the immunogenicity of KBMA *E. coli* HA SD/H1N1-S-OIV, ICR mice were intranasally vaccinated with KBMA *E. coli* over-expressing HA of SD/H1N1-S-OIV or a negative GFP control protein (KBMA *E. coli* GFP) for nine weeks. *E. coil* inactivation was demonstrated by its inability to form colonies on LB agar plates (Fig. 2). A band appearing at approximately 64 kDa was visualized when purified recombinant HA was reacted with the serum obtained from mice immunized with KBMA *E. coli* HA SD/H1N1-S-OIV (Fig. 4A). Moreover, no immunoreactivity against recombinant HA was detected when serum from mice immunized with KBMA *E. coli* GFP were used (Fig. 4A). Furthermore, no band appearing at 64 kDa was detected when purified recombinant GFP was reacted with the serum from mice immunized with KBMA *E. coli* HA SD/H1N1-S-OIV (Fig. 4A). These data indicated that KBMA *E. coli* HA SD/H1N1-S-OIV-immunized mice were able to produce antibodies to HA even a relatively low amount (1.4 μg/10^8 CFU) of HA was produced in *E. coli*.

To examine if antibodies to HA in mouse sera are capable of inhibiting virus-induced hemagglutination, guinea pig erythrocytes were mixed with SD/H1N1-S-OIV in the presence of serum collected from mice three weeks after the first immunization with KBMA *E. coli* HA SD/H1N1-S-OIV or KBMA *E. coli* GFP. As shown in Fig. 4B, the sera from KBMA *E. coli* HA SD/H1N1-S-OIV-immunized mice noticeably reduced hemagglutination titer by approximately 4-fold. To avoid the possibility that naïve serum or PBS exhibits the hemagglutination activity, erythrocytes were incubated with naïve serum or PBS alone in the absence of SD/H1N1-S-OIV. As expected, no hemagglutination activity was observed in the presence of naïve serum or PBS alone (Fig. 4B). To investigate if serum obtained from mice boosted with KBMA *E. coli* HA SD/H1N1-S-OIV shows stronger inhibitory effects on hemagglutination, erythrocytes were mixed with SD/H1N1-S-OIV in the presence of serum obtained from mice three weeks after the second immunization (Fig. 4C). Remarkably, the boosted sera reduced hemagglutination titer by approximately 16-fold (Fig. 4C). These data demonstrated that mice immunized with KBMA *E. coli* HA SD/H1N1-S-OIV produced neutralizing antibodies against SD/H1N1-S-OIV.

Effect of antibody to HA of SD/H1N1-S-OIV on the seasonal H1N1 virus-induced hemagglutination

A computational analysis on the whole HA sequences of 2009 pandemic swine H1N1 strains and seasonal H1N1 strains revealed that positions discriminating the pandemic swine H1N1 from seasonal human strains were located in or near known H1N1 antigenic sites, thus camouflaging the pandemic swine H1N1 strains from immune recognition (35). In agreement with previous observation, we found that antibody to swine origin influenza A (H1N1) HA did not cross-react with HA of a seasonal H1N1 strain. Only purified recombinant HA of SD/H1N1-S-OIV, but not HA of seasonal ATCCH1N1, can be recognized by antibody to swine origin influenza (H1N1) HA (Fig. 5A). Similarly,

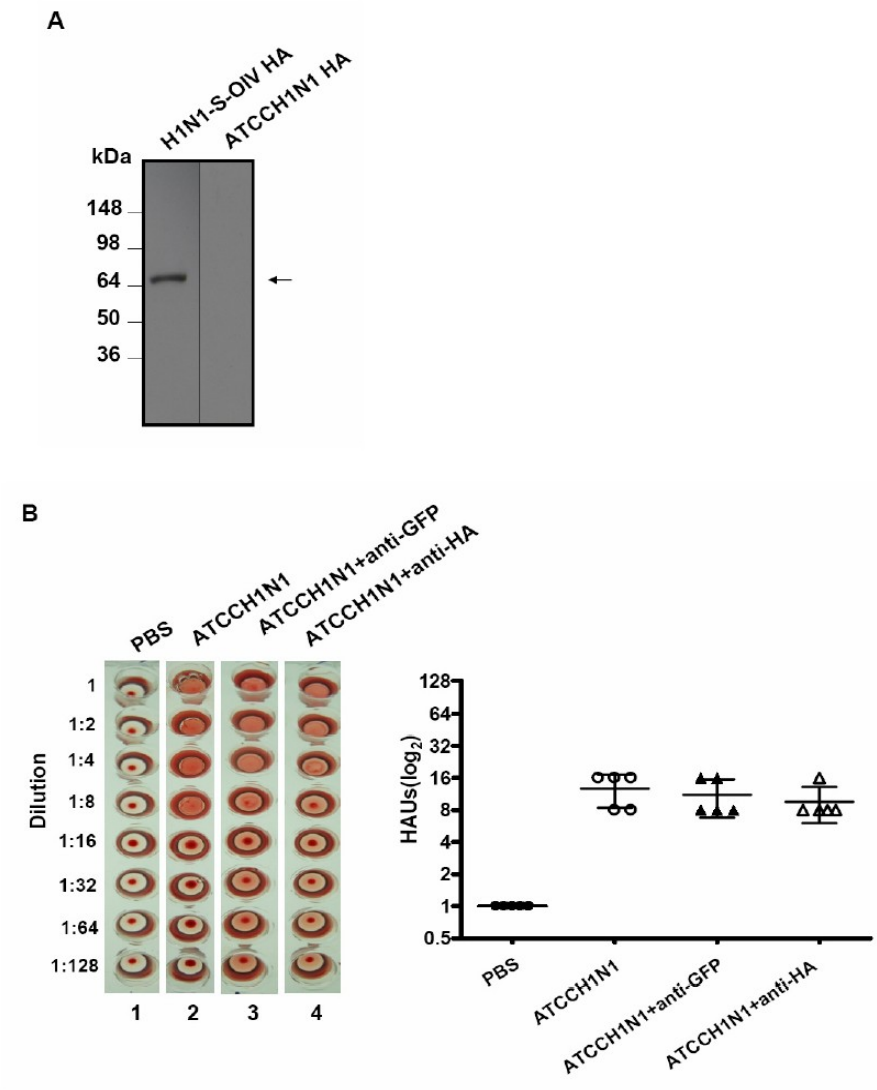

Figure 5. Selectivity of antibodies to SD/H1N1-S-OIV HA and a seasonal H1N1 virus. (A) Recombinant HA (10 μg) of SD/H1N1-S-OIV and ATCCH1N1 was reacted with a commercial antibody to swine origin influenza A (H1N1) HA (1:500 dilution) in Western blot analysis. (B) Inhibition of ATCCH1N1-induced erythrocyte hemagglutination using neutralizing antibody to SD/H1N1-S-OIV HA. The HIA was performed by mixing erythrocytes with PBS (lane 1), PBS plus twofold serial dilution (1-128) of ATCCH1N1 (16 HAU), (lane 2), ATCCH1N1 plus anti-GFP (lane 3) or ATCCH1N1 plus anti-HA (lane 4) as described in Fig. 2 legend. The inhibition of hemagglutination activity was expressed as log2 of HAUs and calculated from six independent experiments.

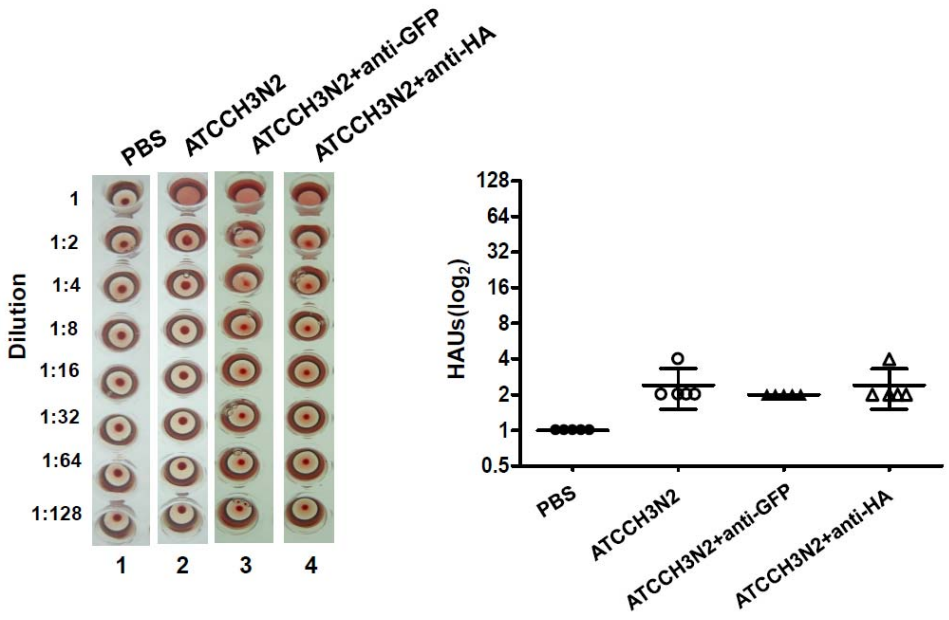

Figure 6. The antibody to HA of SD/H1N1-S-OIV did not inhibit the ATCCH3N2-induced hemagglutination. The HAI was performed as described in Fig. 2 legend. Briefly, erythrocytes were mixed with PBS (lane 1), PBS plus twofold serial dilution (1-128) of ATCCH3N2 (2 HAU), (lane 2), ATCCH3N2 plus anti-GFP or ATCCH3N2 plus anti-HA (lane 4) as described in Fig. 2 legend. The inhibition of hemagglutination activity was expressed as log2 of HAUs and calculated from six independent experiments.

antibody to HA of SD/H1N1-S-OIV did not show inhibitory effects on ATCCH1N1-induced hemagglutination (Fig. 5B). Moreover, serum obtained from mice immunized with KBMA *E. coli* HA SD/H1N1-S-OIV or KBMA *E. coli* GFP fail to neutralize the hemagglutination induced by ATCCH3N2 (Fig. 6)., indicating the specificity of neutralizing antibodies against SD/H1N1-S-OIV-, but not seasonal influenza viruses (H1N1 and H3N2), -induced hemagglutination.

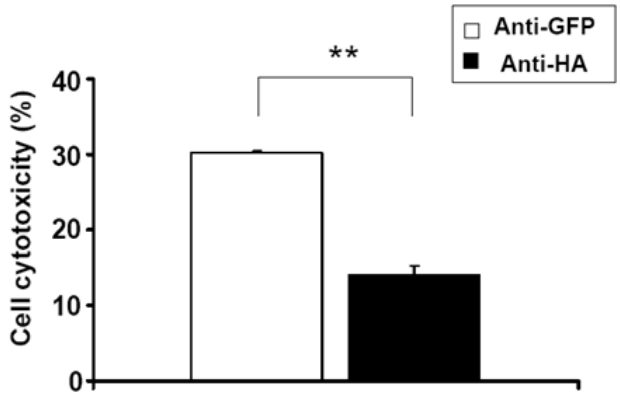

Figure 7. Inhibition of viral infectivity by neutralizing antibodies to HA of SD/H1N1-S-OIV. MDCK cells were infected with a 1:100,000 dilution of SD/H1N1-S-OIV (16 HAU) in the presence of serum from mice immunized with KBMA *E. coli* GFP (anti-GFP) or KBMA *E. coli* HA SD/H1N1-S-OIV (anti-HA) for two days. Cell viability was determined by an ACP assay as described in the Materials and methods. Data are presented as mean ± SE. Student *t*-test was used to assess the significance of independent experiments. The criterion (**p<0.005) was used to determine statistical significance.

Neutralizing activity of antibody to HA of SD/H1N1-S-OIV in mammalian cells

To study if antibody to HA of SD/H1N1-S-OIV possesses the ability to reduce the infectivity of SD/H1N1-S-OIV in mammalian cells, MDCK cells were infected with SD/H1N1-S-OIV overnight in the presence of serum obtained from mice immunized with KBMA *E. coli* HA SD/H1N1-S-OIV or KBMA *E. coli* GFP. The presence of serum from KBMA *E. coli* HA SD/H1N1-S-OIV-immunized mice considerably decreased the cell death (14.05 ± 0.24 %) caused by virus infection compared to that of the same amount of serum from KBMA *E. coli* GFP-immunized mice (30.13 ±1.06%) (Fig. 7). The results evidently illustrated that antiviral activity of neutralizing antibody to HA in the sera of KBMA *E. coli* HA SD/H1N1-S-OIV-immunized mice.

Discussion

The pandemic of H1N1influenza virus highlighted a need to employ alternative approaches for a more rapid generation of vaccines for global immunization in response to impending influenza pandemic. In this study, we took advantages of KBMA microbes as an antigen carrier to simplify and accelerate the production of neutralizing antibodies to influenza virus. Firstly, we produced HA in *E. coli*, allowing rapid expression and subsequent large-scale, cost-effective manufacturing of recombinant HA. Secondly, we used the intact particle of KBMA *E. coli* which contains natural adjuvants (20,36) and over-expressed HA to induce neutralizing antibody responses (37,38) even HA was expressed at relatively low level without post-translational modification in *E. coli*. Different components (e.g. lipopolysaccharide, CpG DNA and mRNA (36) of *E. coli* can activate various receptor-signaling pathways to trigger diverse immune responses including cellular (Th1) and humoral (Th2)

immunity (22). Thus, the KBMA *E. coli* itself may be a natural adjuvant to enhance the immunity of HA that has been expressed in *E. coli*. Thirdly, vaccination with intact particle of KBMA *E. coli* over-expressing HA can eliminate the time-consuming steps required for purification of recombinant HA. In addition, intranasal administration was chosen as an immunization route. The nasal cavity has a large surface area, an excellent blood supply and a highly permeable epithelial membrane (37) for eliciting both local and systemic antibody responses (38,39). Moreover, an immunization route through intranasal administration has been used for the Food and Drug Administration approved influenza vaccines (40). FluMist® (an intranasal seasonal influenza vaccine) is marketed in the US, and NASOVAC™ (a nasal H1N1 vaccine) was launched recently in India (41). Thus, the appeal of needle-free intranasal immunization is evident, especially when mass immunizations are required, and is a very topical subject given the pandemic H1N1 influenza outbreak.

Several studies with bacterially expressed HA proteins based on the H5N1 avian influenza virus have been reported (5,16,42) and one clinical trial with a bacterially expressed fusion protein between the HA fragment and flagellin from *Salmonella typhimurium* type 2 is underway (17), indicating HA is an excellent antigen target for developing influenza vaccines. Although *E. coli* has been used as an expression system to express the HA of H1N1 influenza viruses in the previous studies (2,43), recombinant HA was obtained via complicated purification and refolding procedures. Furthermore, antibodies to a globular head domain of HA rather than full length of recombinant HA were produced. It has been documented that full-length proteins may be superior immunogens to single domains because they more closely mimic the native structure (44). In those studies, animals were administered intramuscularly with more than 7.5 μg of purified HA for antibody production and hemagglutination inhibition (2,43). Although the codon-optimized HA gene had been constructed to enhance the HA production in various expression vectors (45,46) our results showed that vaccination of mice with *E. coli* expressing a relatively low amount (1.4 μg) of HA was capable to generate neutralizing antibodies to HA (Fig. 1).

The influenza virus causes annual epidemics of influenza, largely due to the selection of new variants with mutations in the surface HA. The surface HA determines the antigenic properties of the virus and combines with sialic acid (SA) residues on epithelial cells during cell attachment (47,48). In this study, antibodies to HA of SD/H1N1-S-OIV specifically inhibit SD/H1N1-S-OIV-, but not seasonal H1N1- (Fig. 5) and H3N2-, induced (Fig. 6) hemagglutination, supporting that the pandemic SD/H1N1-S-OIV may derive from mutations in the HA of seasonal H1N1 viruses. The concept of receptor binding domain mutants could be used to develop preemptive vaccines and therapeutic monoclonal antibodies that neutralized new variants (47).

In conclusion, we use the KBMA *E. coli* over-expressing HA as an intranasal vaccine to generate specific neutralizing antibodies to SD/H1N1-S-OIV. The use of KBMA *E. coli* for vaccine production will provide a potent modality to effectively control the rapid transmission of influenza virus in the early phases of the pandemic because vaccination with KBMA *E. coli* SD/H1N1-S-OIV HA has three unique characteristics: 1) expressing full size of HA; 2*)* requiring no purification of recombinant HA and exogenous adjuvants; and 3) producing on a very large scale in a timely fashion. Future studies include evaluating the efficacy of vaccination with KBMA *E. coli* SD/H1N1-S-OIV HA against SD/H1N1-S-OIV in animals and assessing the safety of KBMA *E. coli* that can be accepted by the public, manufacturers and regulators.

Acknowledgements

This work was supported by National Institutes of Health Grants (R01-AI067395-01, R21-R022754-01, R21-I58002-01 and 1R41AR056169-01) and Ministry of Science and Technology (MOST) in Taiwan. We thank Xing-Quan Zhang and Robert T. Schooley for their assistance at virus growth.

Author Contributions

Conceived and designed the experiments: CMH YTL. Performed the experiments: PFL. Analyzed the data: PFL YHW. Contributed reagents/materials/analysis tools: YTL. Wrote the paper: PFL CMH.

1. Thompson, W.W., Shay, D.K., Weintraub, E., Brammer, L., Cox, N., Anderson, L.J., & Fukuda, K. 2003. Mortality associated with influenza and respiratory syncytial virus in the United States. *JAMA* 289: 179-186.
2. Aguilar-Yanez, J.M., Portillo-Lara, R., Mendoza-Ochoa, G.I., Garcia-Echauri, S.A., Lopez-Pacheco, F., Bulnes-Abundis, D., Salgado-Gallegos, J., Lara-Mayorga, I.M., Webb-Vargas, Y., León-Angel, F.O., Rivero-Aranda, R.E., Oropeza-Almazán, Y., Ruiz-Palacios, G.M., Zertuche-Guerra, M.I., DuBois, R.M., White, S.W., Schultz-Cherry, S., Russell, C.J., & Alvarez, M.M. 2010. An influenza A/H1N1/2009 hemagglutinin vaccine produced in Escherichia coli. *PLoS One* 5: e11694.
3. Alvarez, M.M., Lopez-Pacheco, F., Aguilar-Yanez, J.M., Portillo-Lara, R., Mendoza-Ochoa, G.I., García-Echauri, S., Freiden, P., Schultz-Cherry, S., Zertuche-Guerra, M.I., Bulnes-Abundis, D., Salgado-Gallegos, J., Elizondo-Montemayor, L., & Hernández-Torre, M. 2010. Specific recognition of influenza A/H1N1/2009 antibodies in human serum: a simple virus-free ELISA method. *PLoS One* 5: e10176.
4. Girard, M.P., Tam, J.S., Assossou, O.M., & Kieny, M.P. 2010. The 2009 A (H1N1) influenza virus pandemic: A review. *Vaccine* 28: 4895-4902.
5. Biesova, Z., Miller, M.A., Schneerson, R., Shiloach, J., Green, K.Y., Robbins, J.B., & Keith, J.M. 2009. Preparation, characterization, and immunogenicity in mice of a recombinant influenza H5 hemagglutinin vaccine against the avian H5N1 A/Vietnam/1203/2004 influenza virus. *Vaccine* 27: 6234-6238.
6. Kistner, O., Barrett, P.N., Mundt, W., Reiter, M., Schober-Bendixen, S., & Dorner, F. 1998. Development of a mammalian cell (Vero) derived candidate influenza virus vaccine. *Vaccine* 16: 960-968.
7. Reisinger, K.S., Block, S.L., Izu, A., Groth, N., & Holmes, S.J. 2009. Subunit influenza vaccines produced from cell culture or in embryonated chicken eggs: comparison of safety, reactogenicity, and immunogenicity. *J Infect Dis* 200: 849-857.
8. Erlewyn-Lajeunesse, M., Brathwaite, N., Lucas, J.S., & Warner, J.O. 2009. Recommendations for the administration of influenza vaccine in children allergic to egg. *BMJ* 339: b3680.
9. Schwarzer, J., Rapp, E., Hennig, R., Genzel, Y., Jordan, I., Sandig, V., & Reichl, U. 2009. Glycan analysis in cell culture-based influenza vaccine production: influence of host cell line and virus strain on the glycosylation pattern of viral hemagglutinin. *Vaccine* 27: 4325-4336.
10. Liu, J., Shi, X., Schwartz, R., & Kemble, G. 2009. Use of MDCK cells for production of live attenuated influenza vaccine. *Vaccine* 27: 6460-6463.
11. Szymczakiewicz-Multanowska, A., Groth, N., Bugarini, R., Lattanzi, M., Casula, D., Hilbert, A., Tsai, T., & Podda, A. 2009. Safety and immunogenicity of a novel influenza subunit vaccine produced in mammalian cell culture. *J Infect Dis* 200: 841-848.

12. Kuroda, K., Hauser, C., Rott, R., Klenk, H.D., & Doerfler, W. 1986. Expression of the influenza virus haemagglutinin in insect cells by a baculovirus vector. *EMBO J* 5: 1359-1365.
13. Kuroda, K., Groner, A., Frese, K., Drenckhahn, D., Hauser, C., Rott, R., Doerfler, W., & Klenk, H.D. 1989. Synthesis of biologically active influenza virus hemagglutinin in insect larvae. *J Virol* 63: 1677-1685.
14. Fedson, D.S. 2008. NEW technologies for meeting the global demand for pandemic influenza vaccines. *Biologicals* 36: 346-349.
15. Yap,.Y.K., Smith, D.R. 2010. Strategies for the plant-based expression of dengue subunit vaccines. *Biotechnol Appl Biochem* 57: 47-53.
16. Chiu, F.F., Venkatesan, N., Wu, C.R., Chou, A.H., Chen, H.W., Lian, S.P., Liu, S.J., Huang, C.C., Lian, W.C., Chongm, P., & Leng, C... 2009. Immunological study of HA1 domain of hemagglutinin of influenza H5N1 virus. *Biochem Biophys Res Commun* 383: 27-31.
17. Song, L., Nakaar, V., Kavita, U., Price, A., Huleatt, J., Tang, J., Jacobs, A., Liu, G., Huang, Y., Desai, P., Maksymiuk, G., Takahashi, V., Umlauf, S., Reiserova, L., Bell, R., Li, H., Zhang, Y., McDonald, W.F., Powell, T.J., & Tussey, L. 2008. Efficacious recombinant influenza vaccines produced by high yield bacterial expression: a solution to global pandemic and seasonal needs. *PLoS One* 3: e2257.
18. Davis, A.R., Nayak, D.P., Ueda, M., Hiti, A.L., Dowbenko, D., & Kleid, D.G. 1981. Expression of antigenic determinants of the hemagglutinin gene of a human influenza virus in Escherichia coli. *Proc Natl Acad Sci U S A* 78: 5376-5380.
19. Brockstedt, D.G., Bahjat, K.S., Giedlin, M.A., Liu, W., Leong, M., Luckett, W., Gao, Y., Schnupf, P., Kapadia, D., Castro, G., Lim, J.Y., Sampson-Johannes, A., Herskovits, A.A., Stassinopoulos, A., Bouwer, H.G., Hearst, J.E., Portnoy, D.A., Cook, D.N., Dubensky, T.W. Jr. 2005. Killed but metabolically active microbes: a new vaccine paradigm for eliciting effector T-cell responses and protective immunity. *Nat Med* 11: 853-860.
20. Lankowski, A.J., & Hohmann, E.L. 2007. Killed but metabolically active Salmonella typhimurium: application of a new technology to an old vector. *J Infect Dis* 195: 1203-1211.
21. Skoble, J., Beaber, J.W., Gao, Y., Lovchik, J.A., Sower, L.E., Liu, W., Luckett, W., Peterson, J.W., Calendar, R., Portnoy, D.A., Lyons, C.R., & Dubensky, T.W. Jr. 2009. Killed but metabolically active Bacillus anthracis vaccines induce broad and protective immunity against anthrax. *Infect Immun* 77: 1649-1663.
22. Zhang, J., Shi, Z., Kong, F.K,, Jex, E., Huang, Z., Watt, J.M,, Van Kampen, K.R., & Tang, D.C. 2006. Topical application of Escherichia coli-vectored vaccine as a simple method for eliciting protective immunity. *Infect Immun* 74: 3607-3617.
23. Liu, P.F., Nakatsuji, T., Zhu, W., Gallo, R.L., & Huang, C.M. 2011. Passive immunoprotection targeting a secreted CAMP factor of Propionibacterium acnes as a novel immunotherapeutic for acne vulgaris. *Vaccine* 29: 3230-3238.
24. Lo, C.W., Lai, Y.K., Liu, Y.T., Gallo, R.L., & Huang, C.M. 2011. Staphylococcus aureus hijacks a skin commensal to intensify its virulence: immunization targeting beta-hemolysin and CAMP factor. *J Invest Dermatol* 131: 401-409.
25. Liu, P.F., Shi, W., Zhu, W., Smith, J.W., Hsieh, S.L., Gallo, R.L., & Huang, C.M. 2010 Vaccination targeting surface FomA of Fusobacterium nucleatum against bacterial co-aggregation: Implication for treatment of periodontal infection and halitosis. *Vaccine* 28: 3496-3505.
26. Alekshun, M.N., & Levy, S. B. 2006. Commensals upon us. *Biochem Pharmacol* 71: 893-900.
27. Stephenson, I., Zambon, M.C., Rudin, A., Colegate, A., Podda, A., Bugarini, R., Del Giudice, G., Minutello, A., Bonnington, S., Holmgren, J., Mills, K.H., & Nicholson, K.G. 2006. Phase I evaluation of intranasal trivalent inactivated influenza vaccine with nontoxigenic Escherichia coli enterotoxin and novel biovector as mucosal adjuvants, using adult volunteers. *J Virol* 80: 4962-4970.
28. Sur, D., Lopez, A.L., Kanungo, S., Paisley, A., Manna, B., Ali, M., Niyogi, S.K., Park, J.K., Sarkar, B., Puri, M.K., Kim, D.R., Deen, J.L., Holmgren, J., Carbis, R., Rao, R., Nguyen, T.V., Donner, A., Ganguly, N.K., Nair, G.B., Bhattacharya, S.K., & Clemens, J.D. 2009. Efficacy and safety of a modified killed-whole-cell oral cholera vaccine in India: an interim analysis of a cluster-randomised, double-blind, placebo-controlled trial. *Lancet* 374: 1694-1702.
29. Mahalanabis, D., Lopez, A.L., Sur, D., Deen, J., Manna, B., Kanungo, S., von Seidlein, L., Carbis, R., Han, S.H., Shin, S.H., Attridge, S., Rao, R., Holmgren, J., Clemens, J., & Bhattacharya, S.K. 2008. A randomized, placebo-controlled trial of the bivalent killed, whole-cell, oral cholera vaccine in adults and children in a cholera endemic area in Kolkata, India. *PLoS One* 3: e2323.
30. Lu, Q., Zhang, X.Q., Pond, S.L., Reed, S., Schooley, R.T., & Liu, Y.T. 2009. Detection in 2009 of the swine origin influenza A (H1N1) virus by a subtyping microarray. *J Clin Microbiol* 47: 3060-3061.
31. Chen, C.H., Zhang, X.Q., Lo, C.W., Liu, P.F., Liu, Y.T., Gallo, R.L., Hsieh, M.F., Schooley, R.T., Huang, C.M. 2010. The essentiality of alpha-2-macroglobulin in human salivary innate immunity against new H1N1 swine origin influenza A virus. *Proteomics* 10: 2396-2401.
32. Liu, P.F., Haake, S.K., Gallo, R.L., & Huang, C.M. 2009. A novel vaccine targeting Fusobacterium nucleatum against abscesses and halitosis. *Vaccine* 27: 1589-1595.
33. Martin, A., & Clynes, M. 1991. Acid phosphatase: endpoint for in vitro toxicity tests. *In Vitro Cell Dev Biol* 27A: 183-184.
34. Olsen, B., Munster, V.J., Wallensten, A., Waldenstrom, J., Osterhaus, A.D., & Fouchier, R.A. 2006. Global patterns of influenza a virus in wild birds. *Science* 312: 384-388.
35. Masoodi, T.A., Shaik, N.A., Shafi, G., Munshi, A., Ahamed, A.K., & Masoodi, Z.A. 2012. Comparative analysis of hemagglutinin of 2009 H1N1 influenza A pandemic indicates its evolution to 1918 H1N1 pandemic. *Gene* 491: 200-204.
36. Sander, L.E., Davis, M.J., Boekschoten, M.V., Amsen, D., Dascher, C.C., Ryffel, B., Swanson, J.A., Müller, M., & Blander, J.M. 2011. Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. *Nature* 474: 385-389.
37. Henkin, R.I. 2011. Intranasal delivery to the brain. *Nat Biotechnol* 29: 480.
38. Davis, S.S. 2001. Nasal vaccines. *Adv Drug Deliv Rev* 51: 21-42.
39. Yuki, Y., & Kiyono, H. 2009. Mucosal vaccines: novel advances in technology and delivery. *Expert Rev Vaccines* 8: 1083-1097.
40. Bren, L. 2003. West Nile virus: reducing the risk. *FDA Consum* 37: 20-27.
41. Watts, P.J., & Smith, A. 2011. Re-formulating drugs and vaccines for intranasal delivery: maximum benefits for minimum risks? *Drug Discov Today* 16: 4-7.
42. Shen, S., Mahadevappa, G., Oh, H.L., Wee, B.Y., Choi, Y.W., Hwang, L.A., Lim, S.G., Hong, W., Lal, S.K., & Tan, Y.J. 2008. Comparing the antibody responses against recombinant hemagglutinin proteins of avian influenza A (H5N1) virus expressed in insect cells and bacteria. *J Med Virol* 80: 1972-1983.
43. Khurana, S., Verma, S., Verma, N., Crevar, C.J., Carter, D.M., Manischewitz, J., King, L.R., Ross, T.M., & Golding, H. 2010. Properly folded bacterially expressed H1N1 hemagglutinin globular head and ectodomain vaccines protect ferrets against H1N1 pandemic influenza virus. *PLoS One* 5: e11548.
44. Avril, M., Hathaway, M.J., Srivastava, A., Dechavanne, S., Hommel, M., Beeson, J.G., Smith, J.D., & Gamain, B. 2011. Antibodies to a full-length VAR2CSA immunogen are broadly strain-transcendent but do not cross-inhibit different placental-type parasite isolates. *PLoS One* 6: e16622.
45. Mesonero, A., Suarez, D.L,, van Santen, E., Tang, D.C., & Toro, H. 2011. Avian influenza in ovo vaccination with replication defective recombinant adenovirus in chickens: vaccine potency, antibody persistence, and maternal antibody transfer. *Avian Dis* 55: 285-292.
46. Sipo, I., Knauf, M., Fechner, H., Poller, W., Planz, O., Kurth, R., & Norley, S. 2011. Vaccine protection against lethal homologous and heterologous challenge using recombinant AAV vectors expressing codon-optimized genes from pandemic swine origin influenza virus (SOIV). *Vaccine* 29: 1690-1699.
47. Yang, Z.Y., Wei, C.J, Kong, W.P., Wu, L., Xu, L., Smith, D.F., & Nabel, G.J. 2007. Immunization by avian H5 influenza hemagglutinin mutants with altered receptor binding specificity. *Science* 317: 825-828.
48. Hasegawa, H., Ichinohe, T., Strong, P., Watanabe, I., Ito, S., Tamura, S., Takahashi, H., Sawa, H., Chiba, J., Kurata, T., & Sata, T. 2005. Protection against influenza virus infection by intranasal administration of hemagglutinin vaccine with chitin microparticles as an adjuvant. *J Med Virol* 75: 130-136.