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Protection of Mice Against Pandemic H1N1 Influenza Virus Challenge After Immunization with Baculovirus-Expressed Stabilizing Peptide Fusion Hemagglutinin Protein S

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Current influenza vaccines are produced in embryonated chicken eggs. However, egg-based vaccines have various problems. To address these problems, recombinant protein vaccines have been developed as new vaccine candidates. Unfortunately, recombinant proteins frequently encounter aggregation and low stability during their biogenesis. It has been previously demonstrated that recombinantly expressed proteins can be greatly stabilized with high solubility by fusing stabilizing peptide (SP) derived from the C-terminal acidic tail of human synuclein (ATS). To investigate whether SP fusion proteins can induce protective immunity in mice, we produced influenza HA and SP fusion protein using a baculovirus expression system. In *in vitro* tests, SP-fused recombinant HA1 (SP-rHA1) was shown to be more stable than recombinant HA1 (rHA1). Mice were immunized intramuscularly with baculovirus-expressed rHA1 protein or SP-rHA1 protein (2 μg/mouse) formulated with aluminum hydroxide. Antibody responses were determined by ELISA and hemagglutination inhibition assay. We observed that SP-rHA1 immunization elicited HA-specific antibody responses that were comparable to rHA1 immunization. These results indicate that fusion of SP to rHA1 does not negatively affect the immunogenicity of the vaccine candidate. Therefore, it is possible to apply SP fusion technology to develop stable recombinant protein vaccines with high solubility.

Keywords: Pandemic, influenza, hemagglutinin, stabilizing peptide

Introduction

In 1918, the Spanish flu pandemic caused global panic. It was highly infectious and deadly, causing 50 million deaths worldwide. The pandemic was caused by the H1N1 influenza virus [11]. Since then, numerous large and small scale influenza virus epidemics and pandemics have occurred, placing heavy disease burdens on the public, and, in 2009, the H1N1 swine-flu pandemic again received global attention [3, 5]. It is certain that new and unpredictable influenza virus pandemics are going to take place in the future, and the disease burden caused by such a pandemic would be even greater without a fast and an effective prevention strategy.

Vaccines are the most effective means of preventing influenza virus infection. Currently, various influenza virus vaccines have been developed and tested clinically. Most of the commercially available vaccines are produced from embryonated chicken eggs. However, production of egg-based vaccines takes several months and largely depends on the timely production of the eggs [17]. During pandemics, the egg-based system is limited in producing a

large amount of vaccines in a short period of time. Recently, cell-culture-based production of influenza vaccines, which can overcome the limitations of the egg-based system, has been clinically approved. In particular, a protein expression system using recombinant baculovirus-infected insect cells can produce a large amount of subunit vaccine in a short period of time [1]. Accordingly, a baculovirus expression system has been explored and adopted for the production of various subunit vaccines for different pathogens [19−21].

Adjuvants are commonly used to increase the immunogenicity of subunit vaccines. Currently, alum is the most widely used adjuvant and has been employed as a safe and powerful immune enhancer since 1926 [15]. Various commercially available vaccines for a myriad of diseases such as DTP, HPV, tetanus, anthrax, Japanese encephalitis, and hepatitis B are formulated with alum [9]. Despite the use of adjuvants, however, the low solubility and instability of subunit vaccine during the purification and storage process are major problems for recombinant protein vaccines. Furthermore, protein vaccine can be aggregated by external stresses such as heat, and an unstable form of protein can induce unpredictable immune responses that can potentially be harmful to the recipient of the vaccine [2, 16].

Stabilizing peptide (SP) is derived from the C-terminal acidic tail of synuclein (ATS), and recombinant proteins fused with SP have been shown to maintain their proper stable forms under various stresses [6, 10, 12]. According to previous studies, therapeutic protein fused with SP is protected from agitation, heat stress, and repeated freeze-thaw cycles [8, 10, 12, 13]. These studies indicate that SP-fusion improves the stability, while maintaining the original characteristics, of the target protein in *in vitro* conditions. However, how such increases in stability derived through SP-fusion would affect the immunogenicity of recombinant protein vaccines has not been examined.

In this study, we compared the immunogenicity and protection generated by vaccination of mice with recombinant influenza HA1 proteins, either with or without SP-fusion, that were produced *via* a baculovirus expression system.

Materials and Methods

Cloning and Protein Expression

Cloning transfer vector. The globular head domain of the hemagglutinin gene of influenza virus A/New York/3571/2009 (H1N1) strain (rHA1, residues 18-344) was amplified with adaptor primers that had additional restriction enzyme recognition sequence for *Bgl*II (forward) and *Xba*I (reverse) at the 5′ end of each primer. The amplicon was cut with restriction enzymes, and then inserted into baculovirus transfer vector pAcGP67 (BD Bioscience, Franklin Lakes, NJ, USA) with a 6× His-tag added at the C-terminus. The vector for expression of chimeric protein containing the SP sequence (SP-rHA1) underwent additional modification to encode acidic tale sequences (Fig. 1A). Construction of the transfer vectors was conducted in an *E. coli* system. (Sequence information for cloned proteins is listed in Supplementary file.)

Production of recombinant virus and protein production in an insect cell system. The recombinant transfer vectors were transformed to 7.5 × 10^5 cells/ml SF9 cells (BD Bioscience) by the calcium phosphate method and baculovirus was simultaneously infected to the insect cell. Over two consecutive subcultures, we obtained high-titer viruses in the supernatants. For recombinant protein expression, new SF9 cells (2 × 10^6 cells/ml, 200 ml) were infected with the supernatants containing recombinant virus and cultured for 3 days (27°C, 115 rpm), and then the culture media were collected and concentrated by the ammonium sulfate

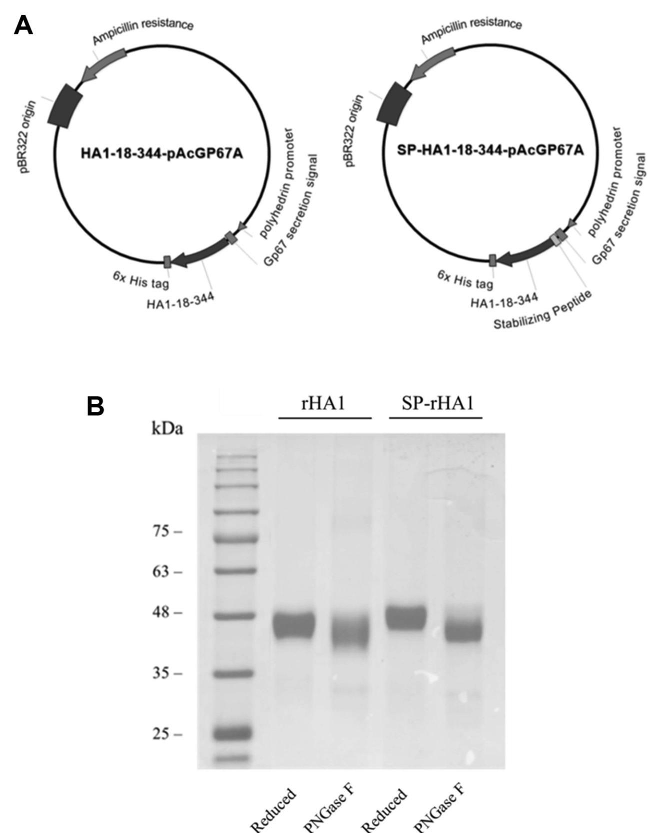

Fig. 1. Cloning and expression of recombinant proteins.

Partial sequences (residues 18−344) of the HA gene from influenza virus A/New York/3571/2009 (H1N1) strain was cloned into a baculovirus transfer vector with modifications of histidine tagging and/or stabilizing peptide fusion (**A**). Recombinant proteins were confirmed by SDS-PAGE analysis in reducing condition, and the status of glycosylation was also confirmed with use of PNGase F (**B**).

precipitation method (7 M ammonium sulfate). The precipitate was re-dissolved in a buffer solution (20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 5 mM imidazole) and then we purified histidine tagged proteins using a nickel column (Nickel beads Cat.1018; ADAR Biotech, USA). The purified protein was eluted from the column with elution buffer (20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 255 mM imidazole) and underwent dialysis in 20 mM Tris-HCl buffer at pH 8.0 containing 10% glycerol. Finally, the dialysate was concentrated upto 1 mg/ml.

Confirmation of the expressed protein. The protein content in each concentrate was measured by bicinchoninic acid assay (Thermo Scientific, USA) and 5 µg of each protein was used for SDS-PAGE analysis in reducing conditions. Briefly, samples were mixed with 5× SDS sample buffer, and 2-mercaptoethanol was added to the final concentration of 0.2%. For verification of proper glycosylation in the baculovirus protein expression system, aliquots of samples were pretreated with PNGase F (Sigma Aldrich, St. Louis, MO, USA) according to the method recommended by the manufacturer. All samples were heated at 100°C for 5 min and loaded on 10% Tris-Acetate gel. After separation, protein bands were visualized by 0.05% Coomassie Brilliant Blue staining.

In Vitro Stability Test Against Freeze/Thaw Stress

Sample preparation. Both recombinant proteins, rHA1 and SP-rHA1, were diluted in PBS (pH 7.4) to a concentration of 30 µg/ml, which is the recommended HA concentration in influenza vaccine, and 500 µl of each solution was placed in a microcentrifuge tube.

Effect of freeze/thaw cycles on recombinant proteins. Samples were tested by repeated cycles of freezing in liquid nitrogen and thawing in a 50°C water bath, both for 10 min. Samples that had completed 20, 40, and 60 cycles were collected and subjected to SDS-PAGE. The gel was photographed and the density of each band was semi-quantified using Image J (NIH, USA) software.

Immunogenicity and Protective Efficacy in a Mouse Model

Mice. Six-week-old female Balb/c mice were purchased from Orient Bio. Mice testing was approved by the Institutional Animal Care and Use Committee (IACUC) at the International Vaccine Institute (IVI; no. 2011-009).

Vaccination. ***Case I.*** Balb/c mice were immunized three times every 2 weeks. Mice were vaccinated intramuscularly with recombinant HA1 protein (rHA1) from H1N1 influenza virus (A/California/04/09) using a baculovirus expression system or rHA1 protein fused with SP (SP- rHA1) with alum (Thermo Scientific, USA) as an adjuvant. Either rHA1 or SP-rHA1 was vaccinated using 2 µg per mouse. Control groups were vaccinated with PBS by intramuscular route. Mice sera were collected 2 weeks after every immunization. ***Case II.*** Balb/c mice were immunized at once, in order to confirm the efficacy of minimal vaccination. Mice were vaccinated in the same manner as Case I (rHA1 group, SP-rHA1 group, and negative control group). Mice sera were collected 2 weeks after immunization.

ELISA. Antibody response in vaccinated mice was determined by ELISA. Briefly, a 96-well plate (NUNC, USA) was coated overnight at 4°C with 2 µg/ml of rHA1 protein diluted in 50 mM $NaHCO_3$ buffer (pH 9.6) or A/California/04/09 H1N1 influenza virus diluted in PBS. Following blocking in 1% BSA (Sigma, USA) diluted in PBS, the plate was loaded with immunized mice sera samples. Mice sera were diluted 1:100 in PBS with 1% BSA. Each of the diluted sera samples was transferred to 5-fold serial dilutions, and the plate was incubated for 1−2 h at 37°C. After that, all wells were incubated with 1:3,000 dilutions of goat anti-mouse horseradish-peroxidase-labeled IgG, IgG1, or IgG2a (Southern Biotech, USA) in secondary antibodies buffer (PBS with 0.1% BSA and 0.05% Tween-20) at 37°C for 1−2 h. The development step was then conducted by using tetramethyl benzidine solution (Millipore, USA). The reaction was stopped with 1N HCl. The absorbance values were measured at 450 nm wavelength.

Hemagglutination inhibition assay (HI assay). One day before performing the assay, mice sera were treated with receptor-destroying enzyme (RDE) (Denka-Seiken, Japan) overnight at 37°C. The RDE was then inactivated at 56°C for 1 h. PBS was mixed with the RDE-treated sera, and the sera were diluted 1:10 in RDE with PBS. The enzyme-treated sera were loaded as 2-fold serial dilutions into a V-bottom 96-well plate (Greiner Bio-one, Germany), and 4 hemagglutination units (HAU) of A/California/04/09 H1N1 influenza virus was added. The plate was incubated for 1−2 h at room temperature; then, 1% chicken RBC (Fitzgerald, USA) in Alsever's solution (Sigma, USA) was added to each well. Hemagglutination was observed after 1 h at room temperature. We pooled the samples and the experiments were performed in duplicates.

Viral challenge. Four weeks after the last immunization, mice were infected with A/California/04/09 influenza virus (KRIBB, Korea) at 10 MLD_{50} dose. The mice were then checked daily for body weight loss and survival rate for 14 days. The proportion of body weight change was calculated on the basis of body weight at 0 dpi. After the virus challenge, weight loss ≥20% was used as the endpoint at which mice were euthanized. Survival rate was determined by deaths and euthanized mice.

Results

Recombinant Proteins Were Expressed Well in the Baculovirus Insect Cell System.

Both rHA1 and SP-rHA1 were successfully expressed in the baculovirus insect cell system. SDS-PAGE in reducing conditions confirmed the presence of these proteins at the expected size, and treatment of PNGase F yielded in smaller bands, indicating proper glycosylation during expression in the eukaryotic system (Fig. 1B).

SP-Fusion Enhanced the Stability of rHA1 Protein Over Repeated Freeze/Thaw Cycle Stress.

After 60 freeze-and-thaw cycles, both recombinant

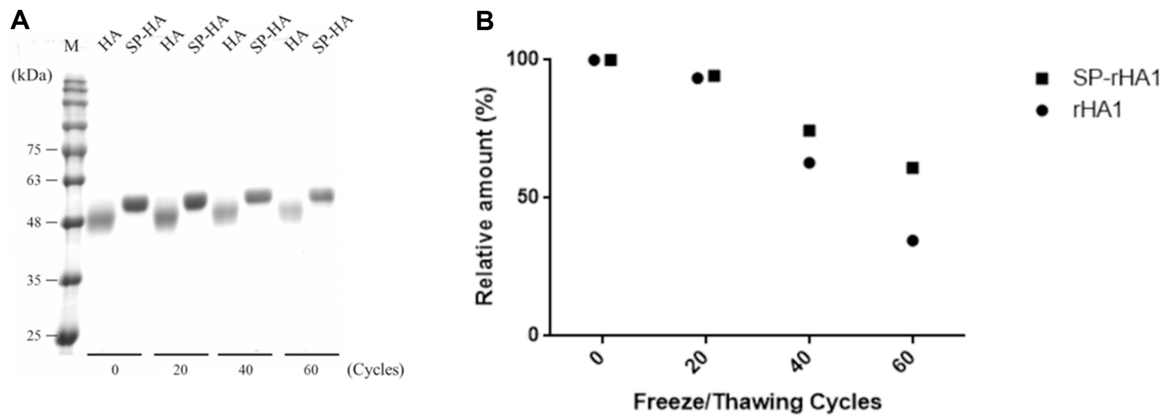

Fig. 2. Freeze/thaw stress on recombinant proteins.
SDS-PAGE analysis of rHA1 and SP-rHA1 showed gradual loss of both proteins over repeated freeze/thaw cycles (**A**). The intensity of each band was semi-quantified using Image J software, and the densities are plotted as a function of freeze/thaw cycles (**B**).

proteins showed a gradual loss of stability over repeated stress (Fig. 2A). However, the densitogram measurements based on the semi-quantified intensities of each band demonstrated a relatively slower loss of SP-rHA1 than of rHA1 (Fig. 2B).

Humoral Immune Response Was Induced by IM Vaccination with rHA1 Protein.

In previous reports, proteins fused with SP were shown to be resistant to agitation, freezing/thawing, and heat stress [10], indicating that the addition of SP grants fusion protein increased stability and longevity for storage. Adding to these previous results, we further confirmed the antiviral activity of the fusion protein (SP-rHA1) compared with, the non-fusion rHA1 protein.

Mice were immunized three times at two-week interval *via* the intramuscular route with 2 μg of rHA1 from H1N1 influenza virus (A/California/04/09) or SP-rHA1 using alum as an adjuvant. Sera were collected from mice 14 days after each immunization, in order to examine the antigen-specific antibody response by ELISA. As shown in Fig. 3, SP-rHA1 immunization induced levels of antigen-specific antibody titers that were as high as the levels induced by rHA1 immunization. Comparing the differences among antibody isotypes, both rHA1 and SP-rHA1 produced higher levels of IgG1 than IgG2a. Therefore, rHA1 and SP-rHA1 immunizations were predicted to induce a Th2-type immune response.

Neutralizing Antibodies Were Induced by rHA1 Protein Against Pandemic H1N1 Influenza Virus.

Next, we confirmed neutralizing antibody titers with an HI assay using mice sera harvested 14 days after the final vaccinations. As shown in Table 1, SP-rHA1 immunization induced a higher level of HI titer than rHA1 immunization. In addition, we further evaluated the serum neutralizing ability generated by SP-rHA1 immunization *via* microneutralization (MN) assay and observed that sera collected 14 days after the final SP-rHA1 vaccination produced an MN titer of 320 (data not shown). These results suggest that SP-rHA1 immunization induces a effective neutralizing antibody response.

rHA1 Was Shown to Protect Against Infection of 2009 Pandemic H1N1 Influenza Virus.

Based on these results, vaccinated mice were challenged with influenza virus to confirm protection against live virus infection. Four weeks after the final immunization, the immunized and negative control mice were challenged intranasally with 10 MLD_{50} H1N1 influenza virus (A/California/04/09). Changes of body weight and the survival rate of the mice were monitored daily, until 2 weeks after the virus challenge. Both the rHA1 and SP-rHA1 groups showed a 100% survival rate (Fig. 4A) with a body weight decrease of less than 10% (Fig. 4B). These

Table 1. HI titer against influenza virus.

Group	HI Titer
PBS	N.D.
rHA1 + Alum	480
SP-rHA1 + Alum	800

A/California/04/09 H1N1 influenza virus-specific neutralizing antibody was examined by HI test.

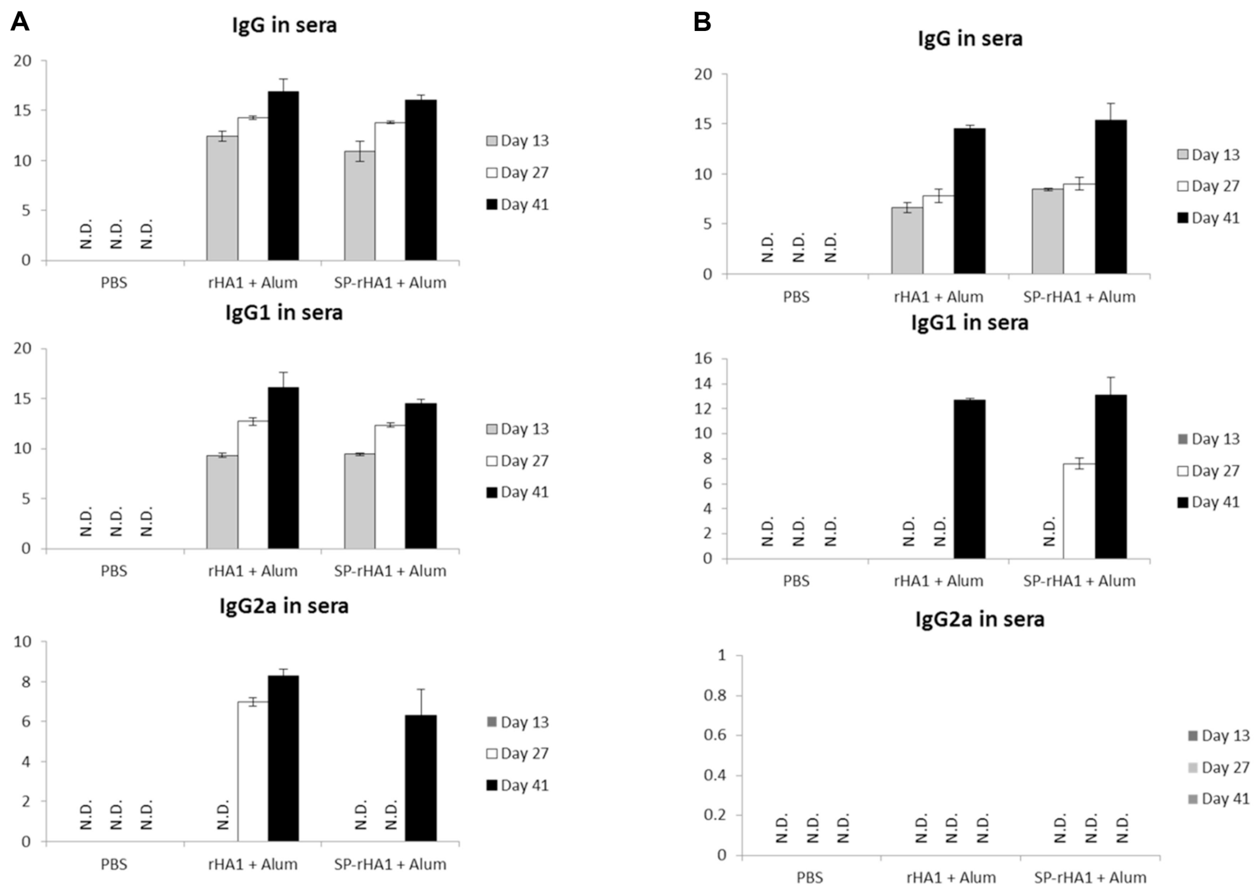

Fig. 3. Influenza antigen-specific antibody responses.

Balb/c mice were immunized with 2 μg of rHA1 protein from A/California/04/09 influenza virus or SP-rHA1 protein with or without an adjuvant. For confirming a systemic immune response, mice were immunized intramuscularly with antigen with alum as an adjuvant (**A**, **B**). Two weeks after every immunization, mice sera were collected and their antibodies were examined by ELISA. IgG, IgG1, and IgG2a antibodies were measured in rHA1 protein-coated (**A**) and virus-coated conditions (**B**).

results show that immunization of mice with rHA1 generated in a baculovirus expression system effectively confers protection against influenza virus infection. Importantly, fusion of SP did not interfere with the ability of SP-rHA1 to induce a strong antiviral activity, as both SP-rHA1 immunization and rHA1 immunization produced comparable levels of protection.

Single Injection of rHA1 Induced a Humoral Immune Response and Protective Activity Against 2009 Pandemic H1N1 Influenza Virus.

In addition, we evaluated the level of protection conferred following a single immunization with rHA1 or SP-rHA1. Mice were immunized intramuscularly with 2 μg of rHA1 from H1N1 influenza virus (A/California/04/09) or SP-rHA1 using alum as an adjuvant. Sera were collected 14 days after the immunization to determine antigen-specific antibody titers using ELISA. Both the rHA1 and SP-rHA1 groups produced high levels of antigen-specific serum antibodies. Animals in these groups also induced higher levels of IgG1 than IgG2a (Fig. 5A). We further performed an HI assay to determine the neutralizing antibody titers; however, no neutralizing ability was detected (data not shown). Next, vaccinated mice were challenged with live influenza virus to examine the level of protection produced after a single immunization. Immunized and control mice were given 10 MLD_{50} of H1N1 influenza virus (A/California/04/09) intranasally. Body weight loss and the survival rate of the mice were recorded daily, until 2 weeks after virus infection. Both the rHA1 and SP-rHA1 groups showed survival rates of about 70% (Fig. 5B). Upon lethal H1N1 influenza virus challenge, mice in the SP-rHA1 and rHA1 groups lost approximately 20% of their body weight (Fig. 5C). In these results, imperfect protection

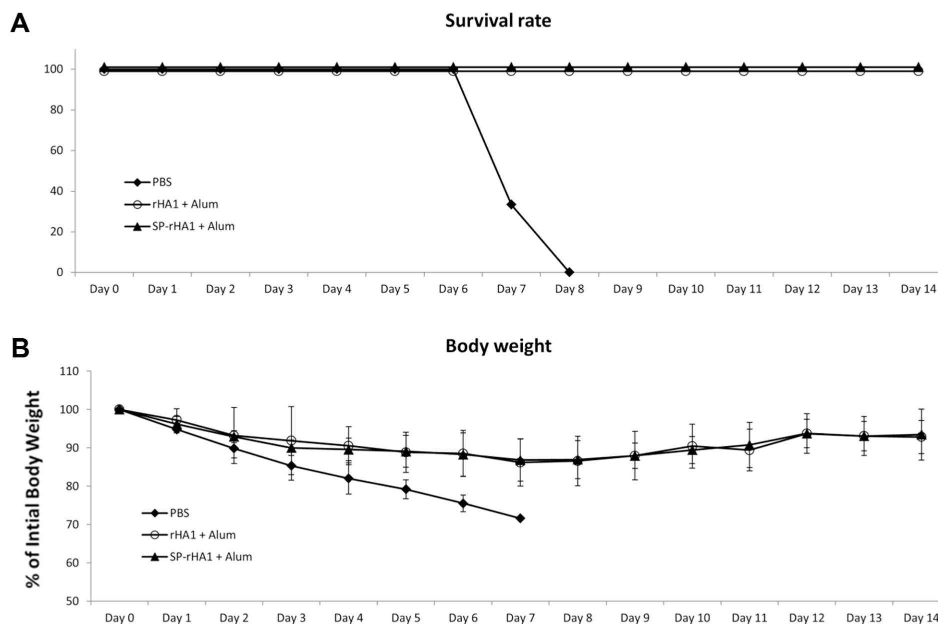

Fig. 4. Viral resistance against influenza virus challenge.

At 4 weeks after the last immunizations, mice were infected intranasally with A/California/04/09 H1N1 influenza virus at 10 MLD_{50}. Their survival rate (**A**) and body weight loss (**B**) were checked every day for 14 days.

activity was shown when mice were vaccinated with a single injection.

Discussion

After the H1N1 Spanish influenza pandemic of 1918, scientists put in great efforts in order to continuously study this field, aiming to providing preventive measures against future influenza virus. Meanwhile, recombinant protein vaccines have gained immense support as a promising tool that can generate strong immune responses and confer protection against viral infections. Accordingly, there has been an increase in the studies evaluating various recombinant protein vaccines in order to prevent future pandemics of viral diseases, and protein vaccines produced from a baculovirus expression system have been studied extensively.

However, recombinant protein vaccines possess difficulties with storage owing to physical disturbances caused by external factors such as heat, freezing, agitation, and shearing [9, 22]. Therefore, these protein vaccines require special measures, such as cold-chain logistics, for transportation and storage [9, 18]. Such constraints render the use of protein vaccines in developing countries as very difficult.

To overcome these obstacles, we utilized SP, found in the acidic tail of human synuclein peptide, as a means of producing more stable and soluble forms of therapeutic proteins *in vitro*. The synuclein family consists of heat-resistant proteins that do not aggregate under heat stress [4, 6, 13], and studies have shown that the acidic tails of the synuclein family are responsible for inducing tolerance against heat, pH, or other environmental stresses [7, 13, 14]. Furthermore, SP functions to increase the stability and solubility of recombinant proteins [6, 8, 10].

In the present study, we confirmed the increase in overall stability of rHA1 as the result of recombinant fusion of SP as SP-rHA1 was shown to be more stable than rHA1 upon experiencing repetitive freeze/thaw cycles. Moreover, to investigate whether the recombinant SP-fusion, which enhances the stability of the fusion protein *in vitro*, affects immunogenicity and protective immunity upon vaccination, mice were immunized intramuscularly with rHA1 or SP-rHA1. We observed that SP-rHA1 elicited HA-specific antibody and a neutralization antibody response that was comparable to that of rHA1, and mice immunized with SP-rHA1 vaccine were completely protected against live influenza virus infection, indicating that SP-fusion does not negatively affect the immunogenicity of fusion proteins. Although we do not possess direct data, we speculate that the presence of SP does not greatly alter the antigen process and presentation of rHA1, given that comparable HA1-specific immunity was generated in mice immunized

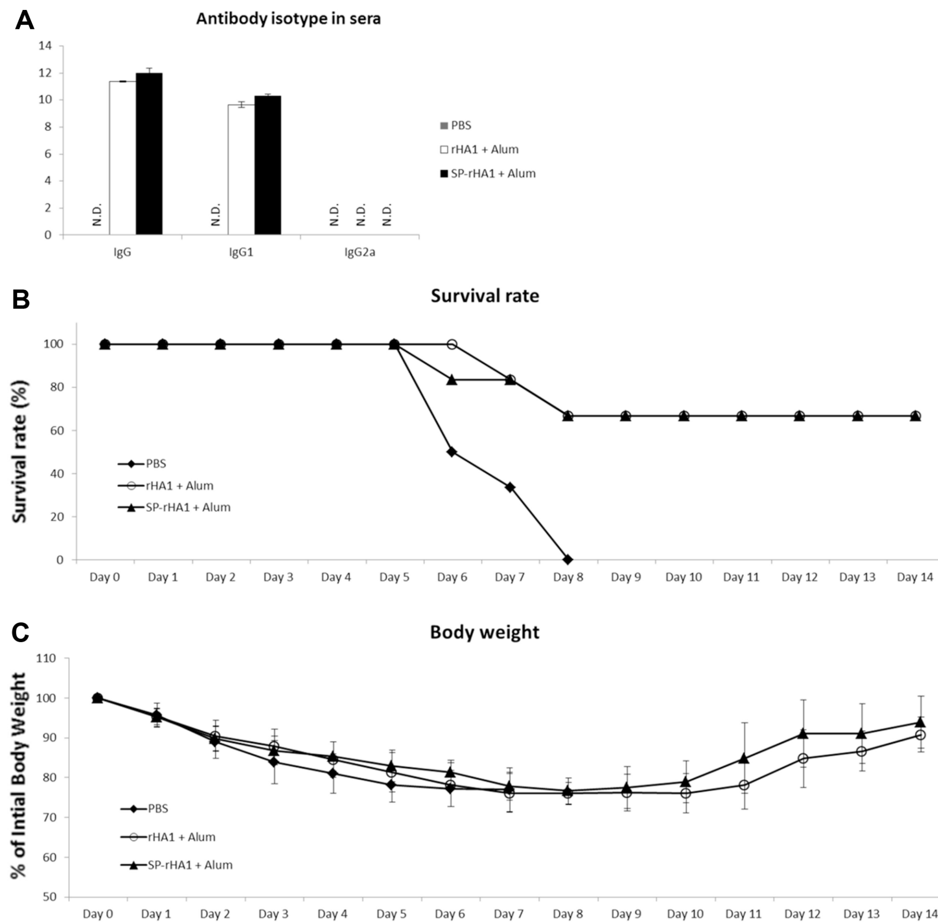

Fig. 5. Immune response with a single injection.

Balb/c mice were immunized once intramuscularly with rHA1 protein or SP-rHA1 protein with alum. Two weeks after immunization, mice sera were collected and their antibodies were examined by ELISA (**A**). Additionally, 4 weeks after immunization, mice were infected intranasally with A/California/04/09 H1N1 influenza virus at 10 MLD_{50} (**B**, **C**). Their survival rate (**B**) and body weight loss (**C**) were checked every day for 14 days.

with SP-rHA1 and in mice immunized with rHA1.

Taken together, we believe that SP-fusion technology could be adopted to generate stable recombinant protein vaccines with high solubility to overcome the current limitations of protein vaccine storage.

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