

Nanoparticles from Plant Saponins as Delivery System for Mucosal Influenza Vaccine

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Abstract This article were studied nanoparticles assembled purified influenza virus hemagglutinin and neuraminidase antigens and low toxicity immunostimulating saponins Gg6 and Ah6, isolated from Kazakhstania plants *G.glabra* and *A.hippocastanum*, in the animal vaccination/challenge experiments as delivery system for mucosal influenza subunit vaccine. Incorporation of influenza virus HA and NA antigens into the structure of nanoparticles significantly increased IgG, IgM and IgA antibody immune responses as well as production of IFN-gamma and IL-2 after single intranasal immunization. Mucosal immunization with nanoparticles possessed HA+NA antigens and saponins Gg6 or Ah6 resulted much better protection of chickens against challenge of highly pathogenic H7N1 avian influenza virus in comparison with immunization of whole virus inactivated vaccine or vaccine mixed with alum hydroxide adjuvant.

Keywords: influenza, nanoparticles, mucosal immunity

1. Introduction

There is an urgent need to develop an influenza vaccine for mucosal immunization to prepare for impending flu pandemic. This vaccine should stimulate an effective systemic and local mucosal immunity and could be administered intranasally as alternative for subcutaneous immunization. In the current socioeconomic and sanitary framework, mucosal vaccination could bring many advantages to existing immunization strategies. It is needle-free, and potentially, easy enough for self-administration. The development of secretory antibodies in mucosa is of great interest, as sites such as the respiratory or gastrointestinal mucosa represent the main gateway of entrance for many pathogens [1]. Mucosal immunity is best achieved by mucosal antigen administration, and recently was the first product based on an intranasal vaccine which has reached the market (Flu Mist®, MedImmune Vaccines, Inc., US, recently re-approved in refrigerated form [2]).

As was described earlier, one of the most successful delivery system for various kinds of antigens of microbial, parasitic or viral origin is immunostimulation complexes (ISCOM) - a unique nanostructure formed by antigens, lipids and immunostimulating saponins of plant origin which combine to stable nanoparticles of about 40-60nm in size [3]. Recent studies have shown that ISCOM nanoparticles incorporated saponin Quil A isolated from the bark of South-American tree *Quillaja saponaria* Molina or purified triterpene saponin QS-21 isolated from Quil A by HPLC fractionation, are highly immunogenic

antigen formulations, which initiate a wide range of antigen-specific immune responses including humoral and CD4/CD8 cell-mediated responses, stimulation of IL and INF-gamma production, and elicitation of mucosal immune response through different routes of immunization: subcutaneous, intranasal and oral [4,5,6].

A number of plants indigenous to Kazakhstan have been found to possess saponins with chemical structure close to Quil A and capably initiate formation of specific nanoparticles through interactions with purified viral or parasite antigens [7,8]. It was shown that nanoparticles incorporated of saponins isolated from Kazakhstania plants stimulated high levels of immune responses and provided good protection against infection through various routes of immunization, including mucosal immunization [7].

In this study we were studied nanoparticles assembled purified influenza virus hemagglutinin and neuraminidase antigens and low toxicity immunostimulating saponins Gg6 and Ah6, isolated from Kazakhstania plants *G.glabra* and *A.hippocastanum*, in the animal vaccination/challenge experiments as delivery system for mucosal influenza subunit vaccine.

2. Materials and Methods

Saponins Gg6 and Ah6 for nanoparticle formation were isolated from two native plants collected in mountainous regions of Kazakhstan: *G. glabra*, *A. hippocastanum*. Crude saponins were extracted from roots and seeds by 95% ethanol extraction and were partially purified from low weight substances by extensive dialysis against

phosphate-buffered saline. Plant extracts were lyophilized and fractionated by HPLC as previously described [9]. HPLC fractions containing highest concentration of saponins were further purified by repeated HPLC fractionation in a linear gradient from 0.1% TFA in water to 80% ACN in water with 0.1% TFA. Before use in immunological experiments, toxicity of saponin preparations was characterized as previously described [9].

Influenza A viruses A/NIBRG-121 XP (H1N1), and A/FPV/Rostok/34 (H7N1) were propagated in 9-day-old chicken embryos. Viral particles were concentrated and purified by centrifugation. Influenza virus hemagglutinin (HA) and neuraminidase (NA) antigens were isolated from purified concentrated viral particles by non-ionic detergent extraction and separated from virus "cores" by centrifugation.

Nanoparticles were prepared by a dialysis technique and mixing of isolated HA and NA antigens, purified saponins and lipids in non-ionic detergent [7,8]. To verify their characteristic structure, samples of nanoparticles were examined by electron microscopy using negative stain with instrumental magnification of 60,000 [10], analyzed by electrophoresis in 12.5% SDS-polyacrylamide gel [11] and amount of protein was determined [12].

Immunostimulation activity of nanoparticles incorporated H1N1 influenza virus HA+NA antigens and plant saponins Gg6 and Ah6 was studied in mice after

intranasal immunization. The titers of specific antibodies were determined by ELISA.

Two-week-old chickens (7 birds per group) were immunized intranasally with various vaccine preparations containing influenza antigens. Two wk after immunization, all groups except the non-immunized, uninfected control group, were infected with influenza virus, strain FPV at a dose of 100 EID₅₀ per bird. Efficiency of vaccination were evaluated during four weeks.

3. Results

Nanoparticles incorporated influenza virus antigens, lipids and saponins Gg6 and Ah6 were analyzed by electron microscopy. As shown in Figure 1, nanoparticles were presented as specific virus-like structures of about 60 nm in size that equal approximately half of size of influenza virus particles.

Toxicity of purified saponins Gg6 and Ah6 has been studied in 3-week-old mice, 1-day-old chickens and 9-day-old chicken embryos. Toxicity of preparations was analyzed in doses 0,1-6,0mg per animal using intranasal, oral, subcutaneous, intramuscular and "in ovo" routes of application. It was shown that both saponins isolated from Kazakhstaniian plants did not induce any toxic reactions in doses exceeded up to 200 times of regular dose of Gg6 and Ah6 saponins used in vaccination experiments (10.0 µg per animal).

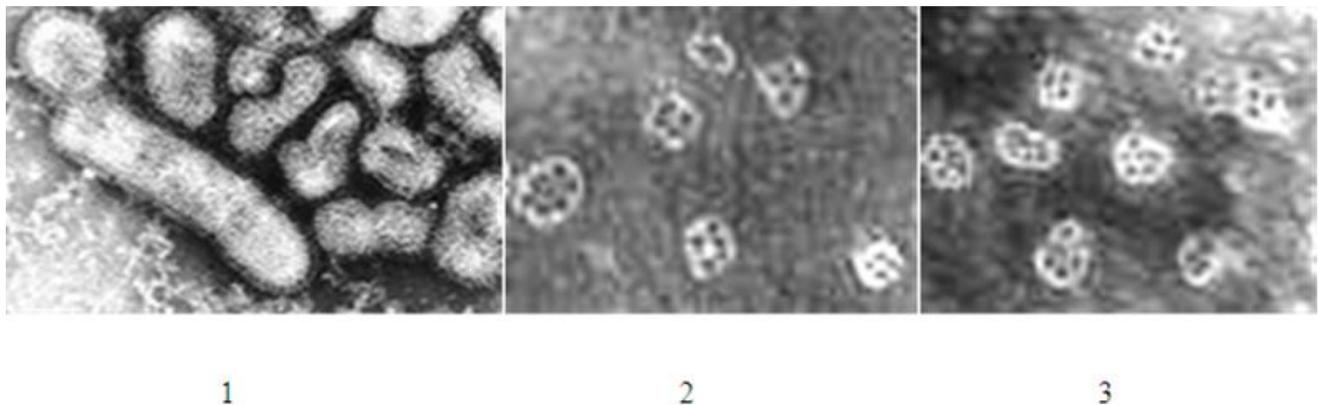


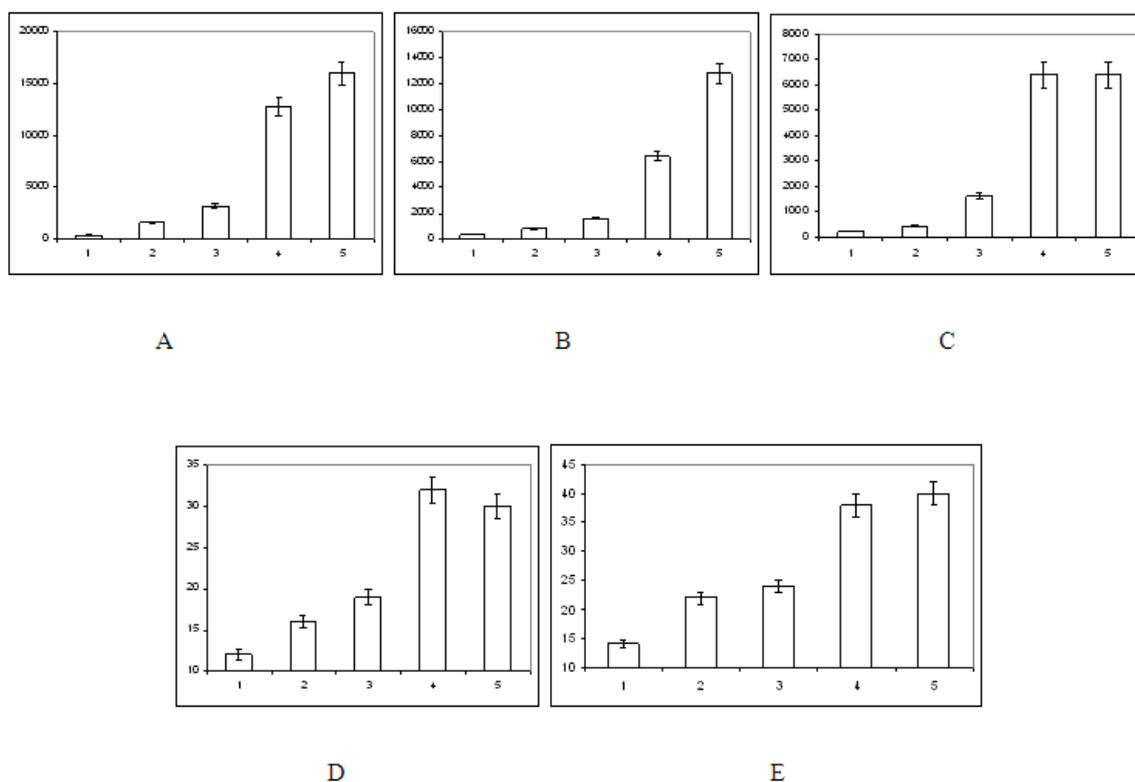
Figure 1. Electron microscopy of purified influenza virus (1) and nanoparticles assembled isolated influenza virus HA+NA antigens and purified saponins Gg6 (2) and Ah6 (3). Magnification - 100 000x

Immunostimulation activity of nanoparticles incorporated H1N1 influenza virus HA+NA antigens and plant saponins Gg6 and Ah6 was studied in mice. Three-week old mice were immunized intranasally by various vaccine preparations: 1) inactivated whole virus influenza vaccine in dose 10.0 µg per mice; 2) inactivated whole virus influenza vaccine mixed with alum hydroxide adjuvant in dose 10.0 µg per mice; 3) nanoparticles incorporated HA+NA antigens and saponin Gg6 in dose 3.0 µg per mice; 4) nanoparticles incorporated HA+NA antigens and saponin Ah6 in dose 3.0 µg per mice. 3 weeks after single immunization titers of IgG, IgM, IgA, IFN-gamma and IL-2 have been measured in mice sera using ELISA kits.

Results of study of immunostimulation activity of nanoparticles incorporated H1N1 influenza virus HA and NA antigens in comparison with immunostimulation activity of H1N1 whole virus inactivated vaccine are

presented in Figure 2. It was shown that single intranasal immunization with nanoparticles containing Gg6 or Ah6 saponins induced much higher titers of IgG, IgM and IgA antibodies as well as IFN-gamma and IL-2 production in comparison with immunization of H1N1 whole virus inactivated vaccine or vaccine mixed with alum hydroxide adjuvant.

Protective capacity of nanoparticles containing H7N1 avian influenza virus HA and NA antigens was studied in chicken vaccination experiments. One-day-old chickens were immunized intranasally with nanoparticles containing isolated HA+NA antigens and saponins Gg6 or Ah6. For comparison, chickens were immunized with whole virus inactivated H7N1 vaccine and vaccine mixed with alum hydroxide adjuvant. Fourteen days after single intranasal immunization, chickens were infected with avian influenza virus, strain A/FPV/Rostock/34 in dose 100 EID₅₀/chicken.



On axis abscissa presented titers of IgG (A), IgM (B) and IgA (C) antibodies (in reciprocal logarithms) and titers of IFN-gamma (D) and IL-2 (E) (in $\mu\text{g}/\text{per ml}$) in mice sera after single intranasal immunization. On axis ordinate presented preparations using for immunization: 1 - placebo; 2 - whole virus inactivated influenza vaccine; 3 - whole virus inactivated vaccine mixed with alum hydroxide adjuvant; 4 - nanoparticles incorporated influenza virus HA+NA antigens and saponin Gg6; and 5 - nanoparticles containing influenza virus HA+NA antigens and saponin Ah6.

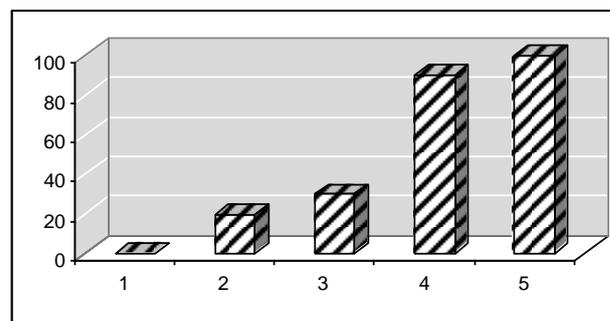
Figure 2. Immunostimulation activity of mucosal H1N1 influenza vaccine containing nanoparticles incorporated HA+NA influenza virus antigens and plant saponins

Results of immunization/challenge experiments are presented in Figure 3. It was shown that single mucosal immunization with influenza subunit vaccine-based nanoparticles containing purified HA+NA antigens and purified saponins isolated from Kazakhstani plants induced high levels of protection against challenge of homologous highly pathogenic avian influenza virus. Immunization with nanoparticles containing Gg6 saponin protected 90% of chickens and same immunization with nanoparticles incorporated Ah6 saponin protected 100% of chickens. In comparison, immunization with whole virus inactivated H7N1 vaccine and same vaccine mixed with alum hydroxide adjuvant protected only 20% and 30% of chickens respectively.

4. Discussion

Mucosal vaccines, especially oral mucosal vaccines, have several advantages over systemic vaccines such as ease of administration without the need for a needle or trained personnel. These features of oral mucosal vaccines make them attractive for mass immunization during pandemics [13]. However, one of the most significant problems associated with oral mucosal vaccines is possible tolerance induction against the orally introduced antigens, because it is generally known that oral administration of soluble proteins dominantly triggers oral tolerance [14, 15]. To overcome this tolerogenic mucosal environment, many efforts have been made to develop effective mucosal adjuvants that can stimulate both innate

and adaptive immunities and are capable of inducing effective mucosal and systemic immune responses.



On axis abscissa presented percentage of protection against challenge, on axis ordinate – preparations used for immunization: 1 – placebo; 2 - whole virus inactivated H7N1 influenza vaccine; 3 - whole virus inactivated H7N1 vaccine mixed with alum hydroxide adjuvant; 4 - nanoparticles incorporated HA+NA antigens and saponin Gg6; 5 - nanoparticles containing HA+NA antigens and saponin Ah6.

Figure 3. Protection against challenge of highly pathogenic A/FPV/Rostock/34 avian influenza virus after mucosal immunization with various vaccine preparations

ISCOMS technique is one of the novel adjuvanted-vaccine systems to meet this demand. ISCOMS has a unique ability to provoke a full range of immune response to protein antigens, which is efficient after both parenteral and oral immunization. It has a unique ability to allow the antigen molecules to enter the endogenous pathway for antigen processing, which in turn to provoke MHC class I-restricted CTL.

In our study, nanoparticles assembled purified influenza virus hemagglutinin and neuraminidase antigens and low toxicity immunostimulating saponins Gg6 and Ah6, isolated from Kazakhstani plants *G.glabra* and *A.hippocastanum* were investigated for their abilities to stimulate humoral and cellular immunity to glycoproteins of H1N1 influenza by intranasal immunization route. Formation of typical structure of cage-like microparticles was verified by electron microscopy. Adequate proportion of the components, extensive dialysis and purification by density gradient ultracentrifugation when necessary, are important factors involved in the preparation of nanoparticles. Our results indicate that intranasal vaccines can induce protective type 1 and secretory IgA responses and may be ideal for inducing both protective mucosal and systemic immunity against influenza virus.

5. Conclusions

Purified low toxicity immunostimulation saponins Gg6 and Ah6 isolated by HPLC fractionation from *G.glabra* and *A.hippocastanum*, plants indigenous to Kazakhstan, may be used for preparation of virus-like nanoparticles containing isolated HA and NA influenza virus antigens.

Incorporation of influenza virus HA and NA antigens into the structure of nanoparticles significantly increased IgG, IgM and IgA antibody immune responses as well as production of IFN- γ and IL-2 after single intranasal immunization.

Mucosal immunization with nanoparticles possessed HA+NA antigens and saponins Gg6 or Ah6 resulted much better protection of chickens against challenge of highly pathogenic H7N1 avian influenza virus in comparison with immunization of whole virus inactivated vaccine or vaccine mixed with alum hydroxide adjuvant.

The presented study results have shown good potential of nanoparticles containing isolated influenza virus HA+NA antigens and saponins Gg6 or Ah6 as a delivery system for mucosal influenza vaccine.

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Statement of Competing Interests

Authors declare that we have no significant competing financial, professional or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

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