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ORIGINAL ARTICLE

Adjuvant activity of multimolecular complexes based on *Glycyrrhiza glabra* saponins, lipids, and influenza virus glycoproteins

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Abstract
Numerous studies have shown that immunostimulatory complexes containing Quil-A saponin and various antigens are effective in stimulating the immune response and can be used as vaccine preparations for animals and humans. However, Quil-A saponin possesses toxicity and haemolytic activity. In the present work, a saponin-containing preparation named "Glabilox" was isolated from the roots of a *Glycyrrhiza glabra* L. plant by high-performance liquid chromatography (HPLC). The results showed that Glabilox has no toxicity or haemolytic activity and can form stable immunostimulatory complexes. Subcutaneous immunization of mice with an immunostimulating complex containing Glabilox and H7N1 influenza virus antigens stimulated high levels of humoral and cellular immunity. Vaccination of chickens with the same immunostimulating complex protected 100% of the animals after experimental infection with a homologous virus. Comparative studies showed that the immunogenic and protective activity of immunostimulatory complexes containing Quil-A and immunostimulatory complexes containing Glabilox are comparable to each other. The results of these studies indicated that *Glycyrrhiza glabra* saponins show great promise as safe and effective adjuvants.

Introduction

All over the world, the emergence of new epidemics and epizootics has become more frequent, along with the continuous spread of acquired immune deficiency syndrome (AIDS) and viral hepatitis, the reappearance of tick-borne encephalitis and haemorrhagic fevers, occasional outbreaks of monkey pox in Africa, and the spread of previously unknown viruses, including new highly pathogenic strains of influenza virus. Thus, it is difficult to overestimate the urgency and importance of research aimed at finding and developing new means of controlling infectious diseases [1–4].

The main and most effective way to control infectious diseases is preventive vaccination. Therefore, research into the development and improvement of vaccines and search for agents that increase their efficacy and safety are highly relevant and occupy one of the leading places in present-day biology and medicine [5–7].

The emergence of a new generation of vaccines is associated with modern advances in molecular biology and biochemistry: obtaining preparations based on individual purified antigens of natural origin or those synthesized using recombinant DNA technology and creating new types of vaccines based on the principles of reverse genetics and the use of vector systems (recombinant viruses, plasmids, etc.) [5–9]. However, despite the advantages of the new generation of vaccines, such as increased safety and manufacturability, compared to traditional vaccines, the vast majority of preparations based on purified viral proteins or nucleic acids integrated into certain carriers have insufficient immunogenicity and are unable to induce a complete immune response. This effect is due to several factors, but it primarily results from differences in the recognition of natural infectious agents and purified viral antigens by immunocompetent cells [10–13].

To increase the immune response and improve the recognition of antigens in vaccines, special additives (adjuvants) are used to stimulate various parts of the immune system and improve the protective properties of vaccines [14, 15].

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The most promising adjuvants include triterpene saponins, which are biologically active glycosides of plant and animal origin [16–18]. In addition to the fact that saponins have their own immunostimulating activity, these compounds are also able to bind lipids and glycoprotein antigens due to hydrophobic-hydrophilic interactions and change their supramolecular organization, forming virus-like nanostructures, thereby improving the presentation of the antigen [19].

The most actively studied preparation based on triterpene saponins is a multimolecular complex of purified antigens, lipids and Quil-A saponin isolated from the bark of the South American tree *Quillaja saponaria* M. [20–23].

The first vaccine based on immunostimulating complex technology was a vaccine against equine influenza, created by ISCOTEC AB and Mallinckrodt in 1989 [24]. Based on the QS-21 preparation isolated from saponin Quil A, commercial vaccines against feline leukaemia (Quilvax-FelV and Leucocell (Smith Kline & French Laboratories), *Borrelia burgdorferi*, which causes canine disease (Quilvax-L), and the causative agent of bovine mastitis (Quilvax-M) have been developed [25]. Currently, Novavax (Sweden), Biensector Brenntag (Denmark), CSL (Australia) and Hawaii Biotech Inc. (USA) [26–30] are developing new vaccines for veterinary and medical purposes based on immunostimulating complex technology.

This article describes studies of the immunostimulating activity of nanostructured complexes created based on purified antigens of influenza virus, lipids, and the saponin-containing preparation Glabilox isolated from the roots of *Glycyrrhiza glabra* L. plants, growing in the territory of the Republic of Kazakhstan. The isolation of Glabilox was performed by high-performance liquid chromatography (HPLC) and identification by electrospray ionization mass spectrometry (ESI-MS). The immunogenic properties of the nanostructures containing Glabilox were compared with those of a commercial aluminium hydroxide adjuvant and Quil-A saponin-containing immunostimulating complexes during the immune response to antigens of avian influenza virus after immunization.

Materials and methods

Solutions and reagents

The following reagents were used: acetonitrile, trifluoroacetic acid (for gradient chromatography; Sigma Aldrich), phosphate-buffered saline (PBS; Amresco), Bradford reagent (Amresco), petroleum ether, diethyl ether, HCl, NaOH, cholesterol (Sigma Aldrich), lecithin from egg yolk (Serva), ethanol, and the non-ionic detergent MESK. Deionized water was obtained using an E-Pure (Barnstead) system.

Virus

The influenza virus strain A/FPV/Rostock /34 (H7N1) was provided by the State Collection of the Ivanovsky Institute of Virology, Moscow, Russia. Influenza virus was cultured in the allantoic cavities of 11-day-old chicken embryos that were free of specific antibodies. Cultivation was carried out for 24 hours at 37 °C. The titre of the virus after cultivation was 10^8-10^9 EID50/ml. The virus was concentrated and purified by differential ultracentrifugation [31]. Purified, concentrated virus was resuspended in a minimal volume of PBS and stored at 4 °C. The haemagglutinating activity of the virus was determined according to standard methods using a 0.75% suspension of chicken erythrocytes or guinea pig erythrocytes [32]. The protein concentration was determined by the Bradford method at a wavelength of 595 nm [33]. To isolate the haemagglutinin and neuraminidase of the influenza virus, the purified and concentrated virus-containing suspension was treated with a 5% solution of the non-ionic detergent MESK, followed by ultracentrifugation and dialysis [31].

Plant materials

All plant material was collected in the territory of the Republic of Kazakhstan. The roots of *G. glabra* L. were chopped to obtain particles of 2-3 mm in size. To destroy the saponin-sterol complexes, the micronized roots were treated with petroleum ether and boiled for 2 hours. The ratio of the mass of plant material to ether was 1:3. Extraction of saponins from plant material was carried out with methanol or ethanol with a ratio of plant material to solvent of 1:5. Saponins were precipitated from the resulting extract with diethyl ether. For comparison and as a standard commercial saponin, Quil-A (Brenntag Biosector, Denmark) was used.

Animals

BALB/c mice at 1 month of age, weighing 18-22 g, and chickens were used in the experiments. All animals were provided and maintained in the animal facility of M. Aikimbayev's Kazakh Scientific Centre for Quarantine and Zoonotic Diseases. Animal care and all manipulations were carried out in accordance with the guidelines established by the Ministry of Education and Science of the Republic of Kazakhstan and were approved by the Ethical Committee of the Research Institute of Microbiology and Virology, Almaty, Kazakhstan.

Cell culture

Madin-Darby canine kidney cells (MDCK) were provided by the Biological Safety Research Institute, Gvardeiskiy, Kazakhstan. The cells were cultured in DMEM (Dulbecco's

modified Eagle medium, Gibco) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Gibco).

High-performance liquid chromatography

Chromatographic separation of the saponin-containing extract of the *G. glabra* L. root was performed using Agilent Technologies™ high-performance liquid chromatography (HPLC), comprising an HPLC pump and a PDA detector scanning at 254 nm. The HPLC conditions were as follows: column, Zorbax C18 (250 mm × 9.4 mm, 5 µm); mobile phase, acetonitrile (A) and water containing 0.1% (v/v) ??trifluoroacetic acid?? (TFA) (B); gradient, 0 min, 0:100; 40 min, 80:20; 50 min, 80:20 (A:B, v/v); flow rate, 1 ml/min. Saponin-containing fractions were collected in the range of 40-45% eluent B. The collected fraction was lyophilized.

Foaming reaction

For the foaming reaction, 5 ml of 0.1 N hydrochloric acid solution was poured into one tube, and 5 ml of 0.1 N sodium hydroxide solution was poured into another tube. Two to three drops of the studied preparation were added to both tubes and shaken vigorously. An equal volume of foam in both test tubes indicated the presence of triterpene saponins. If the foam in the tube with alkali exceeded the other in volume and durability, it was concluded that steroid saponins were present [34].

Chromatography-mass spectrometry analysis

Mass spectrometry detection was performed using a Shimadzu LCMS-8040 instrument with an electrospray ionization (ESI) source. The ESI source was set to positive ionization mode. The parameters in the source were set as follows: capillary voltage, 3.0 kV; source temperature, 120 °C; desolvation temperature, 275 °C; cone gas flow, 50 L h^{-1}; desolvation gas flow, 600 L h^{-1}. For full-scan MS analysis, the spectra were recorded in the m/z range of 200–2000. Sample separation was performed using a Zorbax C18 analytical column (4.6 x 150 mm, 5 µm) in a gradient of 0.1% TFA and 0-80% acetonitrile.

Haemolytic assay

Erythrocytes obtained from the blood of healthy chickens were used to determine the haemolytic activity of the extracts. The analysis was performed by the standard method using a 1% suspension of erythrocytes [35].

Test of saponin toxicity

The toxicity test was conducted on BALB/c mice weighing 15–20 g, chickens aged 10–20 days, and 10-day-old chicken embryos. The investigated preparations were injected subcutaneously into mice and chickens, and chicken embryo preparations were injected into the allantoic cavity. The volume of all injected samples was 200 µl. The saponins were dissolved in sterile PBS at the appropriate concentrations to deliver 62.5, 125, 250, 500, and 1000 µg per animal. Sterile PBS was administered as a control. The animals were observed for 14 days. The toxicity of the preparations was assessed based on mortality, weight loss, and hair loss.

Preparation of immunostimulating complexes

Immunostimulating complexes created on the basis of viral antigens, saponins and lipids were formed by extensive dialysis. A solution containing egg phospholipids (Serva), cholesterol (Sigma), and the investigated saponins in a 1:1:1 ratio was prepared using a 5% solution of the non-ionic detergent MESK. Purified glycoproteins of influenza virus, also dissolved in 5% MESK, were added to the resulting solution. The mixture was dialyzed against PBS, pH 7.2 [31] and the structure and size of the resulting immunostimulating complexes were determined by electron microscopy with negative contrast staining and magnification of 1:100,000 [36].

Immunization

Animals were immunized with whole influenza virus virions, purified glycoprotein antigens of influenza virus (haemagglutinin and neuraminidase), and the same influenza virus antigens mixed with aluminium hydroxide adjuvant and immunostimulating complexes containing glycoprotein antigens of influenza virus, lipids and saponins. The preparations were injected subcutaneously. The dose of whole influenza virus virions administered to each animal was 30 µg, the dose of glycoprotein antigens was 15 µg per animal (as purified antigen and as part of immunostimulating complexes; the dose of injected saponins was 15 µg per animal).

Quantification of IgA, IgG, IgM, IL-2, IL-4, IL-10 and IFNγ

Antibody titre and cytokine levels were determined by a standard method [37]. The analysis was performed in plastic immunoplates with sensitized antigens and species-specific horseradish-peroxidase-labelled antibodies. Orthophenylenediamine was used as a substrate. The ELISA results were assessed by spectrophotometry at a wavelength of 492 nm. The antibody titre was defined as the highest antibody

dilution at which the absorption level was higher than the cutoff line. For IgA, IgG, and IgM determination, commercial preparations of antibodies against specific classes of immunoglobulins labelled with horseradish peroxidase (Southern Biotechnology Associates, Inc., USA) were used.

The level of interleukins was measured by ELISA in accordance with the manufacturer's recommendations (Invitrogen, USA). For each determination, an individual mouse serum without traces of haemolysis was used. Before collecting the data, the standards were measured to check the adequacy of the calibration curve.

Protective activity

One-week-old chickens (10 chickens per group) were immunized with the following preparations: whole inactivated influenza virus virions, purified glycoprotein antigens of influenza virus, and the same glycoproteins mixed with aluminium hydroxide adjuvant and immunostimulating complexes containing antigenic glycoproteins of influenza virus, lipids and saponins. The virus dose was 30 μg per animal, the dose of isolated purified antigens was 10 μg per animal, and the dose of saponins was 15 μg per animal. All preparations were injected subcutaneously. Two weeks after immunization, the experimental groups of animals were infected with influenza virus strain FPV at 10 times the 50% lethal dose (10 LD_{50}) per animal. Animals in the control group were not immunized and were not infected. The protective efficacy of the studied drugs was evaluated within four weeks.

Micro-neutralization test

Micro-neutralization assays were performed on Madin-Darby canine kidney cells (MDCK). Before testing, mouse serum samples were subjected to heat treatment at 56 °C for 30 min. The treated sera were stored at -20 °C until use. To obtain a cell monolayer, MDCK cells (30,000-45,000 cells per well) were seeded in 96-well flat-bottomed plates with DMEM (Gibco). The resulting cell monolayer was used for 3 days. Twofold serial dilutions of the sera were prepared in DMEM (Gibco) after an initial tenfold dilution. The prepared serum dilutions were incubated with the 50% infectious dose for tissue culture ($TCID_{50}$) of the virus for 2 hours at 37 °C in the presence of 5% CO_2. After incubation, the mixture of serum with virus was added to MDCK cells in DMEM (Gibco) supplemented with 10% heat-inactivated FBS and trypsin (Gibco). The resulting mixture was incubated at 37 °C with 5% CO_2. After 3 hours of incubation, the DMEM (Gibco) was refreshed in each well. The cells were observed for 3 to 5 days for the presence of a cytopathic effect. The micro-neutralization titre was defined as the highest dilution at which the integrity of the cell monolayer was maintained [38].

Statistical analysis

Differences between tested samples were evaluated using analysis of variance (ANOVA). Student's *t*-test was used to assess differences between tested samples and control samples. Values for all parameters were expressed as the mean ± standard deviation (SD). *P*-values less than 0.05 were considered statistically significant.

Results

Isolation of triterpene saponins from *G. glabra* L

Saponin-containing extract was obtained from the *G. glabra* L. root by the ethanol extraction method. Isolation of saponin-containing compounds from the resulting extract was performed by sequential fractionation using HPLC. The presence of saponins in the collected fractions was detected based on foaming, and the identification of the substance (Glabilox) in the final fraction was performed by the HPLC-ESI-MS method (Fig. 1). The results showed that the mass-to-charge ratio (m/z) of the selected saponins is 826, which corresponds to triterpenic saponins. The resulting saponin-containing drug was named Glabilox.

Study of the haemolytic activity of Glabilox

The haemolytic activity of Glabilox was compared with that of Quil-A saponin (Fig. 2). The concentration of Quil-A saponin that leads to the haemolysis of 50% of chicken red blood cells (HD_{50}) was found to be 250 μg/ml. Glabilox in the range of concentrations studied did not show any significant haemolytic activity.

The *in vivo* toxicity of Glabilox

The toxicity of Glabilox was studied in comparison with that of Quil-A saponin in experiments on mice, chickens and chicken embryos. Determination of toxicity in terms of animal weight loss and the integrity of the hair coat did not give statistically significant data. The toxicity of the investigated preparations was evaluated by determining the LD_{50}. The results showed (Table 1) that Glabilox did not display toxicity in the investigated concentration range in any of the animal models used. In contrast, Quil-A saponin showed marked toxicity *in vivo*: the 50% lethal dose was 187.5 μg per animal in mice (LD_{50}), 225.2 μg per animal in chickens, and 225.8 μg per animal in chicken embryos.

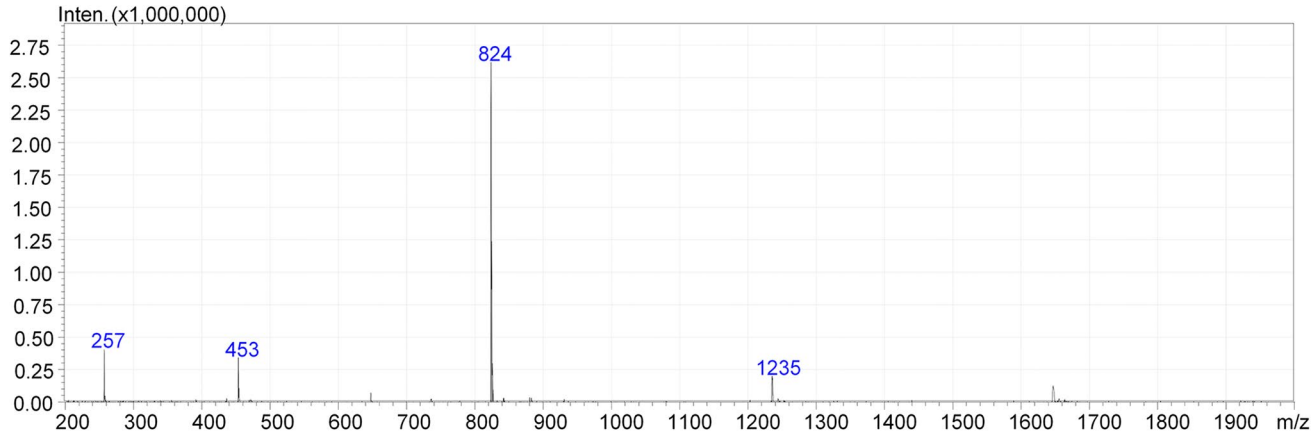

Fig. 1 Mass-to-charge ratio of the Glabilox preparation

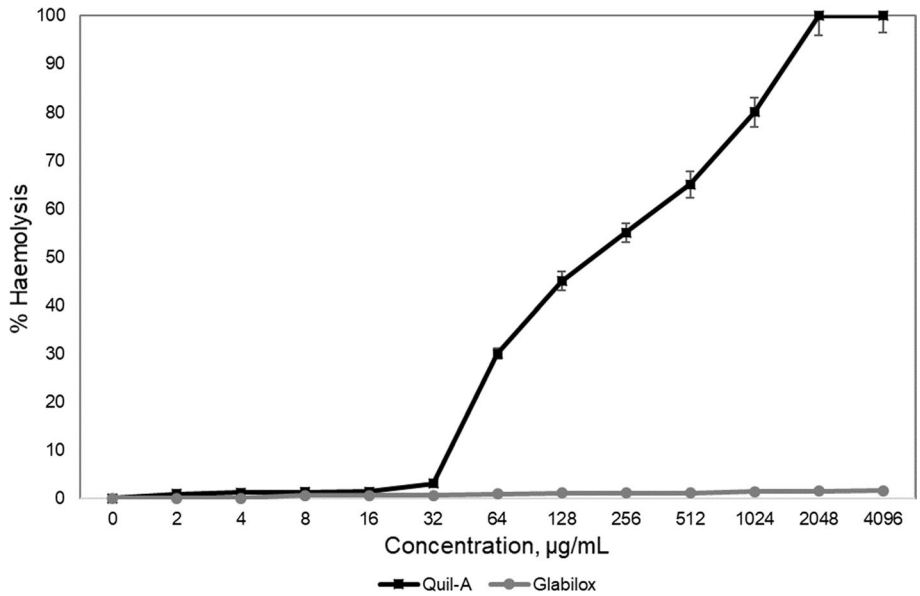

Fig. 2 Haemolytic activity of Glabilox and Quil-A saponins. Data are presented as the mean value ± SD (n = 3)

Table 1 Toxicity of the investigated saponins in *in vivo* experiments

Preparation	LD_{50} (μg/animal), mice*	LD_{50} (μg/animal), chickens *	LD (μg/animal), chicken embryos *
Quil-A	187.5 ± 33.1	225.2 ± 66.1	225.8 ± 66.1
Glabilox	>1000	>1000	>1000

*Variation is indicated as the SD of the mean of three replicates

Structure of Glabilox-based immunostimulating complexes

The structure of the immunostimulating complexes containing lipids, purified influenza virus glycoproteins, and Glabilox was compared with that of a similar immunostimulating complex containing lipids, purified influenza virus glycoproteins, and Quil-A saponin.

As shown in Fig. 3, the use of Glabilox makes it possible to form immunostimulating complexes that are similar in structure to those containing Quil-A. The resulting complexes had an ellipsoidal shape with a diameter of 60-80 nm.

Effect of Glabilox-containing immunostimulating complexes on the synthesis of various classes of immunoglobulins

The immunostimulating properties of the complexes containing Glabilox, lipids, and purified glycoproteins of influenza A/FPV/Rostock/34 virus (H7N1) were investigated by injecting them into BALB/c mice. For comparison, mice were immunized with micelles of H7N1 influenza virus

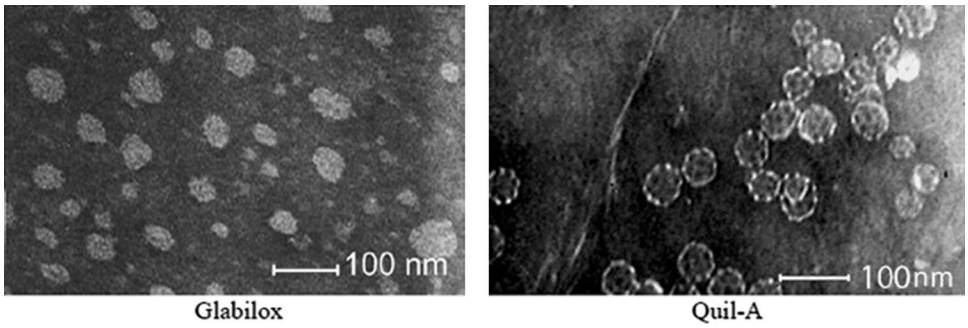

Fig. 3 Immunostimulating complexes composed of plant saponins, lipids, and purified glycoproteins of influenza virus. The structure and size of the nanocomplexes were determined using electron microscopy with negative contrast staining, using an instrumental magnification of 1: 100,000

glycoprotein antigens, micelles of H7N1 influenza virus glycoprotein antigens mixed with aluminium hydroxide adjuvant, whole virus particles, or immunostimulating complexes based on Quil-A saponins. As a negative control, animals were injected with PBS (placebo). Blood from the immunized mice was collected 7 days after the second immunization. The amount of virus-specific IgA, IgG, and IgM antibodies in the blood serum was determined by ELISA.

As shown in Fig. 4, subcutaneous immunization of mice with the Glabilox-based immunostimulating complexes induced a high titre of IgA, IgG, IgM antibodies that was comparable to that obtained by immunization with immunostimulating complexes containing Quil-A saponins. The titres of these classes of immunoglobulins after immunization with the complexes containing Glabilox were 2 times higher than those obtained by immunization with whole virions of influenza virus A/FPV/Rostock/34 (H7N1) and 3-4 times higher than those obtained by immunization with micelles of viral glycoproteins mixed with aluminium hydroxide and with those same antigens without aluminium hydroxide.

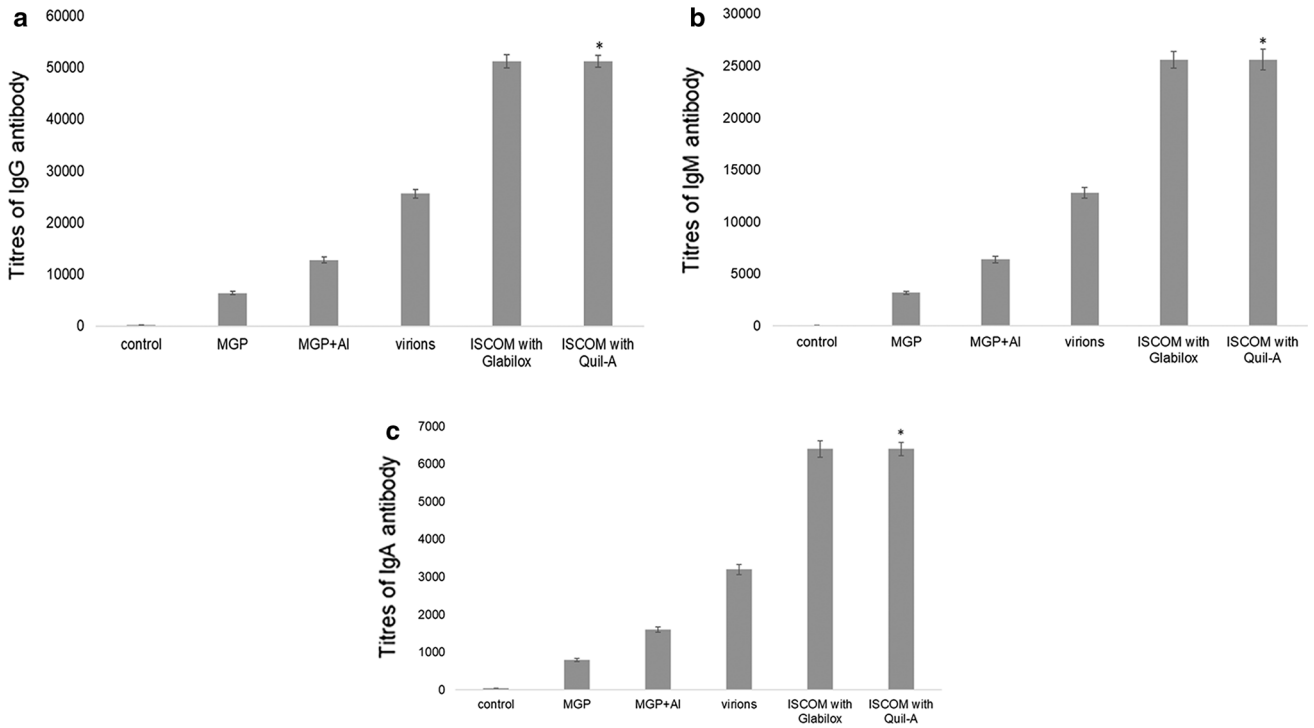

Fig. 4 The levels of IgA (a), IgG (b), and IgM (c) in the serum of immunized mice. Error bars represent the SD for n = 10. *, Statistically significant difference ($p < 0.05$) compared to micelles of glycoproteins. a., b., and c. MGP + Al, micelles of glycoproteins in combination with aluminium hydroxide; MGP, micelles of glycoproteins. Titre of the negative control, < 1:200

Stimulation of cellular immunity factors by immunostimulating complexes containing Glabilox

Cellular immune responses were examined by measuring the level of various cytokines in the serum of immunized mice. BALB/c mice were immunized with immunostimulating complexes containing Glabilox and purified glycoproteins of influenza virus A/FPV/Rostock/34 (H7N1), an immunostimulating complex containing the same glycoproteins and Quil-A, micelles of influenza virus H7N1 glycoproteins in admixture with aluminium hydroxide, or micelles of influenza virus H7N1 glycoproteins without the adjuvant. As a negative control, the animals were injected with PBS (placebo). Preparations were administered subcutaneously twice to each animal with an interval of 3 weeks. The serum was sampled at 7 days after the booster immunization, and the levels of IFN-γ and IL-2 (Th1) and IL-4, and IL-10 (Th2) were determined by ELISA.

The results showed (Fig. 5) that the immunostimulating complexes containing Glabilox induced high levels of the studied cytokines, comparable to those obtained by immunization with Quil-A-containing immunostimulating complexes. All of the immunostimulating complexes that were tested induced the synthesis of significantly higher levels of cytokines IFN-γ, IL-2, IL-4 and IL-10 than were induced by immunization with micelles of H7N1 influenza virus glycoprotein mixed with aluminium hydroxide or without adjuvant.

Protective activity of immunostimulating complexes based on Glabilox and purified glycoproteins of influenza virus

The ability of immunostimulating complexes containing Glabilox and purified glycoproteins of influenza virus A/FPV/Rostock/34 (H7N1) to protect chickens against experimental infection with influenza virus was tested.

For comparison, chickens were immunized with immunostimulating complexes containing Quil-A and influenza A/FPV/Rostock/34 (H7N1) antigens, whole virus particles, and antigen micelles of the same strain with and without aluminium hydroxide. As a negative control, chickens were injected with PBS (placebo). All preparations were administered subcutaneously.

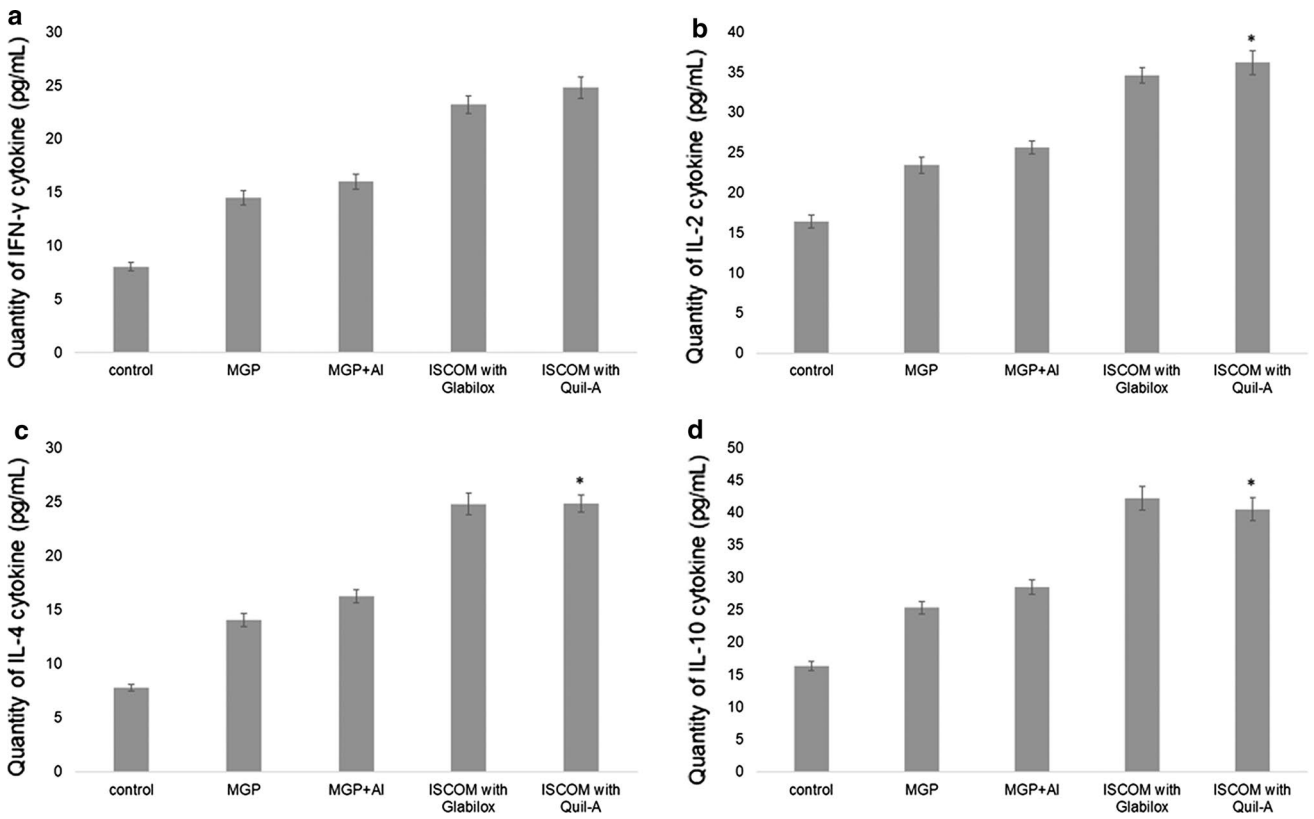

Fig. 5 The level of cytokines in the serum of mice one week after booster immunization. Cytokine concentrations were determined using kits from Invitrogen. Error bars represent the SD for n = 10. *, Statistically significant difference ($p < 0.05$) compared to the micelles of glycoproteins. MGP + Al, micelles of glycoproteins in combination with aluminium hydroxide; MGP, micelles of glycoproteins

Fourteen days after the single immunization, the chickens were infected with the A/FPV/Rostock/34 (H7N1) virus at a dose of 10 LD_{50} per chicken. Specific morbidity or death of chickens was observed for 14 days after the infection.

The results showed that, in chickens immunized with immunostimulating complexes containing saponins and antigens of influenza virus A/FPV/Rostock/34 (H7N1), the survival rate after infection with the homologous virus was 100% (Fig. 6). In the experimental groups in which chickens were immunized with micelles of glycoproteins of influenza virus A/FPV/Rostock/34 (H7N1) without an adjuvant, in the mixture with aluminium hydroxide, and with whole virions, the survival rate was 40%, 50% and 70%, respectively. The survival rate of chickens immunized with PBS was 0%; the survival rate of unimmunized and uninfected chickens was 100%.

Data on the protective effectiveness of the investigated immunostimulating complexes were evaluated by measuring micro-neutralization antibody titres against influenza virus (Fig. 7). All of the immunostimulating complexes investigated induced much higher micro-neutralization titres compared to those induced by immunization with micelles of H7N1 influenza virus glycoprotein antigens without adjuvant and in combination with aluminium hydroxide.

Discussion

Modern adjuvants used in the development of vaccine preparations should significantly increase the immunogenicity of the administered antigens and promote a long-term and sustained immune response while being as safe as possible and causing no toxic, inflammatory, or allergenic reactions after immunization.

Currently, several compounds of different classes are known to be capable of increasing the immune response. These compounds include oil adjuvants, aluminium hydroxide, polysaccharides, lipopolysaccharides, polyelectrolytes, polynucleotides, proteins, some toxins, and a number of preparations of plant origin [39–42]. However, effective and promising adjuvants are those that meet the requirements for immunogenicity and safety. These adjuvants include a number of triterpene saponins isolated from various plants [16, 17].

Triterpene saponins are glycosides whose structure contains hydrophilic and hydrophobic domains. Such an organization of saponin molecules enables these compounds to interact with hydrophobic lipid molecules and amphipathic glycoprotein molecules, which leads to the formation of supramolecular structures.

The most studied is the saponin-containing preparation Quil-A, obtained from the bark of a South American tree, *Quillaja saponaria Molina* [20–23]. Studies with Quil-A have shown that when triterpenic saponins are mixed with lipids and antigenic glycoproteins of different origins, multimolecular immunostimulating complexes called ISCOMs are formed. The resulting nanostructures have a spherical shape with a diameter of approximately 40 nm and are highly stable [35].

ISCOMs have high adjuvant activity and are able to stimulate the development of a specific long-term immune response. These complexes induce cellular, humoral, and local secretory immunity and facilitate the recognition of antigens by antigen-presenting cells, which greatly increases the efficiency and rate of the immune response [15, 16, 43].

Triterpenic saponins are found in the plant *G. glabra* L., which has been widely used in traditional medicine since ancient times [44, 45]. Along with glycyrrhizin, the main saponin, more than 400 other triterpenoids and flavonoids with therapeutic, immunostimulating, antiviral, and antibacterial activity have been isolated from *G. glabra* L. [46]. However, there are still many compounds that have not been fully studied and that may be promising in terms of therapeutic and immunostimulating activity.

In the present work, a saponin-containing fraction was isolated and purified from the root of a Kazakhstani *G. glabra* L plant. Using HPLC/MS, it was found that the m/z ratio of the isolated fraction is 826. The resulting saponin-containing preparation was named Glabilox.

Despite the widespread use of saponin-containing preparations in various industries, medicine, and veterinary applications, many of these preparations are toxic due to their high content of alkaloids, flavonoids, essential oils, tannins, and other substances. In addition, the saponins themselves have surface activity, which gives them haemolytic properties. This is consistent with studies on Quil-A, which showed that some of the compounds of which it is composed have various degrees of toxicity and haemolytic activity [21, 47, 48]. The current study showed that Glabilox has no toxicity or haemolytic activity in the selected dose range. The ability of Glabilox to form multimolecular complexes in mixture with lipids and viral glycoproteins, similar in structure to immunostimulating complexes containing Quil-A, was shown using electron microscopy.

The results revealed that immunostimulating complexes containing Glabilox, lipids, and glycoproteins of H7N1 influenza virus stimulated the synthesis of high titres of IgA, IgG and IgM. The ability of immunostimulating complexes containing viral antigens and Glabilox to stimulate the formation of high levels of cytokines associated with Th-1 (IFN-γ, IL-2) responses and Th-2 (IL-4, IL-10) was also established. The level of immune response induced by those containing Glabilox after subcutaneous immunization of mice was comparable to that induced by immunostimulating complexes containing Quil-A. In addition, the response significantly exceeded that induced by micelles of viral

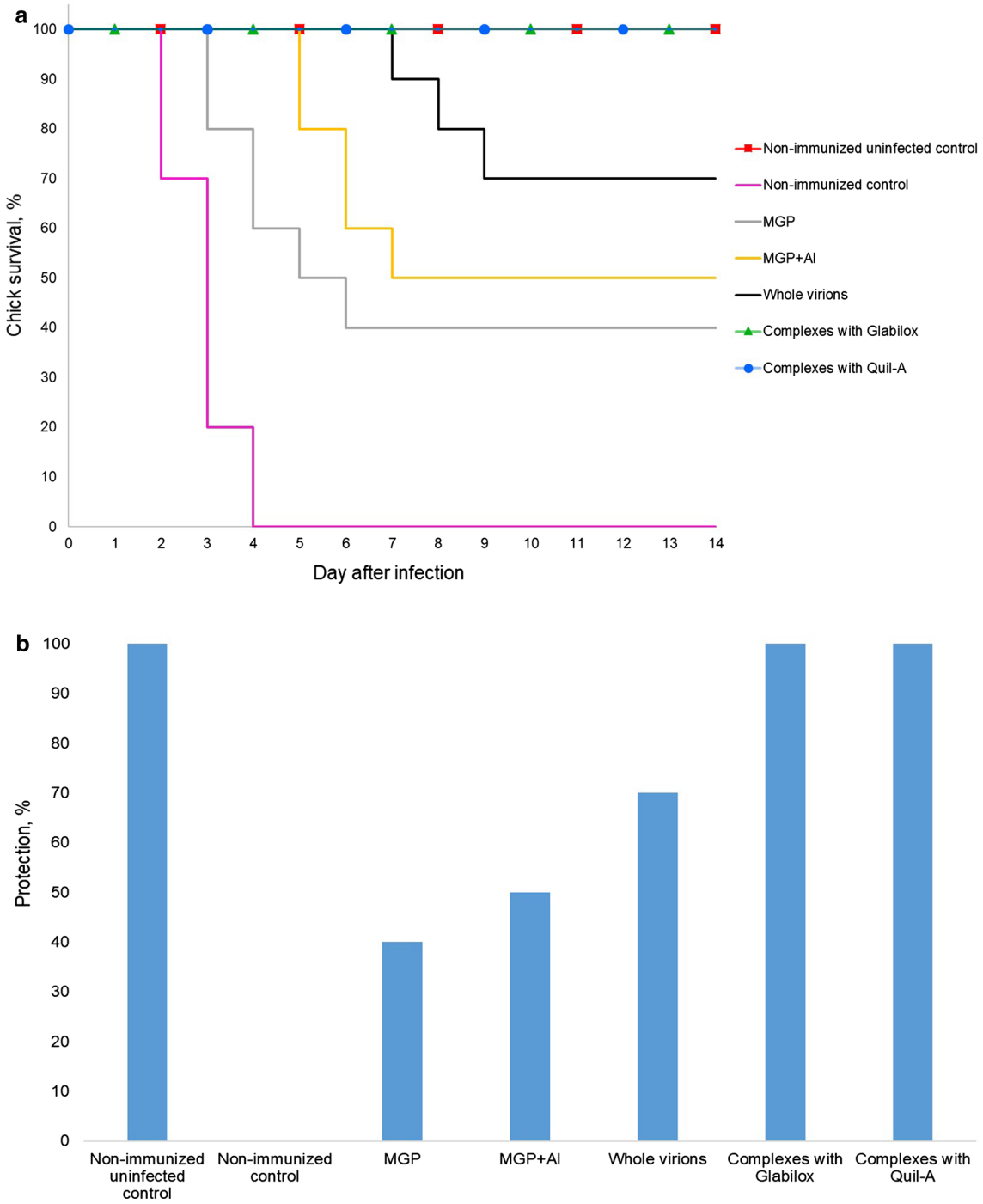

Fig. 6 Survival curve (a) and protective effect of immunostimulatory complexes (b) against influenza virus infection in chickens. MGP + Al, micelles of glycoproteins in combination with aluminium hydroxide; MGP, micelles of glycoproteins

glycoproteins, glycoproteins mixed with aluminium hydroxide adjuvant, or whole virion particles.

The protective efficacy of immunostimulating complexes containing glycoproteins of H7N1 influenza virus with Glabilox or Quil-A was the same and exceeded the protective properties of micelles of viral glycoproteins, micelles mixed with aluminium hydroxide, and whole viral particles. Notably, Glabilox is not toxic and has no haemolytic activity, unlike Quil-A saponin-containing preparations.

In general, the results presented her demonstrate the great potential of *G. glabra* L. triterpenic saponins as adjuvants. Glabilox isolated from the root of *G. glabra* L. displays no

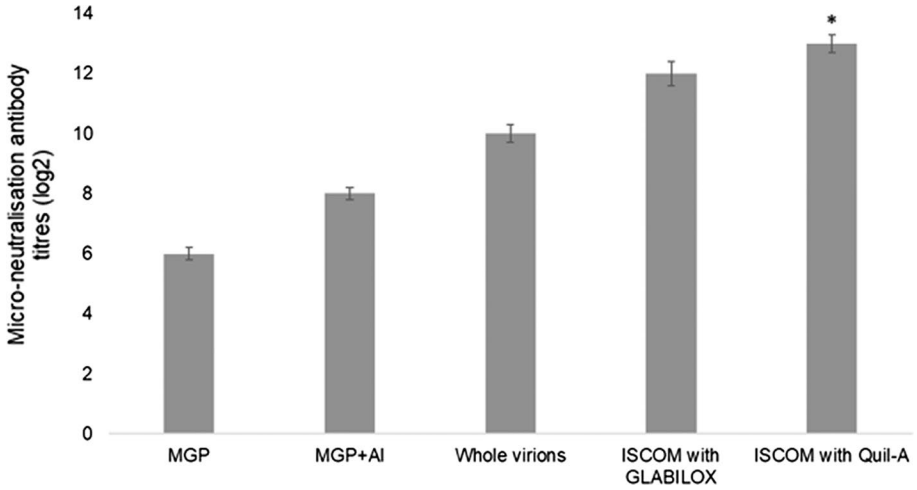

Fig. 7 Micro-neutralization antibody titres of immunostimulating complexes against influenza virus infection. Error bars represent the SD for n = 10. *, Statistically significant difference ($p < 0.05$) compared to the micelles of glycoproteins

toxic properties. Immunostimulating complexes containing Glabilox and purified glycoproteins of H7N1 influenza virus significantly increase the production of virus-specific antibodies, stimulate the synthesis of cellular immunity factors, and enhance the protective immune response, making Glabilox a very promising adjuvant for enhancing the effectiveness of vaccine preparations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent Not applicable.

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