VIRUS-LIKE IMMUNOSTIMULATORY COMPLEXES BASED ON PLANT SAPONINS: PREPARATION, QUALITY CONTROL, ADJUVANT PROPERTIES

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1. INTRODUCTION

Despite significant efforts of national health systems, the World Health Organization, international foundations, the problem of infectious diseases as one of the leading factors of mortality does not lose its urgency. The emergence of such new viruses in the human population as Covid-19, monkeypox, multidrug resistance tuberculosis, *Staphylococcus aureus*, the spread of malaria indicate the need to develop new versions of immunobiologicals.

One of the directions to improve the effectiveness of existing vaccines, to obtain vaccines and immunobiologicals with new properties is the development and implementation of new adjuvants. In 2021, the World Health Organization approved the first antimalarial vaccine Mosquirix (GlaxoSmithKline) containing saponins in virus-like immunostimulatory complexes (ISCOM) as adjuvant. The anti-Covid-19 vaccine Nuvaxovid (Novavax) has approved in many countries. Adjuvanted therapeutic and prophylactic drugs against *Staphylococcus aureus*, tuberculosis, *Clostridium difficile*, genital herpes, *Klebsiella pneumoniae* (GlaxoSmithKline), respiratory syncytial virus, malaria, Ebola virus (Novavax) are at different stages of clinical trials. Saponin-containing ISCOM adjuvants stimulate innate and adaptive cellular immunity, as well as a humoral response characterized by the production of all IgG isotypes, thus, the formation of a mixed Th1/Th2 type of immune response observed. The use of natural and recombinant proteins of bacterial or viral pathogens adjuvanted with ISKOM leads to a pronounced cellular response, which is not characteristic of inactivated drugs, the introduction of which leads mainly to a humoral response [1, 2].

Saponins are triterpene glycosides obtained from plant sources. A characteristic biological feature of saponins is their hemolytic properties. The hydrophobic part of the saponin molecule integrates into the cell membrane. Steric interference of these complexes causes curvature of the membrane, leading to the formation of pores in the membrane. This makes saponins toxic and prevents their use for parenteral administration. The saponins in ISCOM are shielded by cholesterol molecules, do not interact with the cell membrane, and are not toxic. The saponins activate only in the course of metabolic processes in the immune cell that phagocytized the ISCOM-antigen complex [2].

It is possible to obtain and purify ISCOM using a number of biotechnological methods [3]. Previously, ISCOM containing saponins of *Quillaja saponaria* and *Polemonium caeruleum* obtained and purified by liquid chromatography (SEC). This laboratory method has a number of disadvantages, the main one being technical limitations in industrial scale [4]. The preparations containing *Polemonium Caeruleum, Cyclamen europaeum* cause systemic immune response when administered twice intranasally in animals and can be considered as promising mucosal adjuvants, including in combination with ISCOMs [5, 6, 7].

In the production of immunobiologicals, the main task is to standardize the dosing of active components and assess the content of intermediate reaction products in order to guarantee safety. For ISCOM-containing preparations, due to their natural plant origin, the main challenge is to determine the dose of saponins. Nuvaxovid vaccine (Novavax) contains 42.5 μg of fraction A and 7.5 μg of fraction C of *Quillaja saponaria* extract [8]. The level of residual detergent determined by HPLC. The residual concentration of saponins not incorporated into the virus-like particle estimated in a hemolytic reaction [4].

The objectives of this work were to develop a technology for industrial production of ISCOMs based on *Quillaja saponaria* saponins, to create a method for determining the concentration of saponins, detergents based on HPLC and to evaluate the adjuvant properties of the preparation using Covid-19 (SARS CoV-2) antigen and influenza virus when immunizing mice.

2. MATERIALS AND METHODS

The work performed in a laminar flow box, observing the rules of sterility. Approaches to minimize bacterial contamination used: dishes, hoses, FSB, and water were sterilized and depyrogenated. Raw materials weighed on Ohaus Adeventurer scales. The detergents used were Sodium Lauroyl Sarcosine (Amerco, #0719-500G), Cholesterol (PanReac A0807, 0050), Lecithin (PanReac A0893, 0100), Saponin Quilaja saponaria (PanReac #A2542, 0100). The resulting mixture transferred to a tangential filtration-concentration system with a Sartorious VivaFlow 100 kDa module. The product (ISCOM adjuvant Matrix-V) was filtered through a 0.22 μm Minisart NML filterhead (Sartorius) and stored at +4°C. Freezing is not allowed. The particles have pronounced opalescent properties.

For preparation of ISCOM-antigen the antigens of trivalent inactivated split influenza vaccine containing 15 μg of hemagglutinin of each of vaccine strains of influenza viruses A/Victoria/2570/2019 (H1N1)pdm09; A/Cambodia/e0826360/2020 (H3N2); B/Washington/02/2019 (B/Victoria); B/Phuket/3073/2013 (B/Yamagata) without adjuvant. Preparations containing a similar concentration of antigens but without the ISCOM adjuvant used as positive control. The negative control group received the ISCOM adjuvant.

The antigens of the Covid-19 strain also used to prepare the ISCOM antigen preparation. Pools of strain hCoV-19/Russia/Moscow171619-031221/2021, (EPI_ISL_8920444), Omicron 1, BA.1 were prepared on Vero E6 cell culture, underwent a freeze-thaw cycle, centrifuged at 4000g for 10 min, filtered through 0.45 μm filter nozzles, Minisart NML (Sartorius). The preparation was concentrated using 100 kDa centrifuge concentrators (Sartorius), according to the manufacturer's instructions. The resulting concentrated fraction inactivated with β-propiolactone at a concentration of 1:1000 v/v. The residual infectivity of the inactivated fractions confirmed by infecting the Vero E6 cell culture. Residual infectivity was absent for all samples. Complete Freund's adjuvant (Sigma) was used as a comparison adjuvant.

2.1. Immunization of animals

BALB mice weighing 18-20 gr. (SRC VB "VECTOR", Rospotrebnadzor, Russia) used to immunize the animals. Five animals in each group. Inactivated antigen was administered to animals intramuscularly twice at intervals of 3 weeks at 0.1 ml/animal in a mixture with ISCOM adjuvant 1:1. Virus-like immunostimulatory complexes based on *Quillaja saponaria* saponins at a concentration of 160 μg/ml used as adjuvant. The study protocol for using laboratory animals was approved by the Bioethics Commission No. 1 of the Rospotrebnadzor State Research Center Vector (protocol of State Research Center Vector /03-04.2021).

2.2. Neutralization assay

Vero E6 cell culture grown in a 96-well plate under conditions according to the culture passport. The studied mice sera were heated at +56°C for 30 min, then serial double dilutions in DMEM medium were prepared, starting from dilution 1:10. A working concentration of the virus with a titer of 3 lg CPD50/0.1ml was prepared in advance. A mixture of serum dilutions and the working dilution of the virus in equal volumes was prepared. The mixture incubated for 1 h at room temperature, then added to the wells of a 96-well plate with a monolayer of Vero E6 cell culture and incubated for 4 days at 37°C, 5% CO_2. The result counted visually by the presence of CPD over 50% of the monolayer after staining with gentian violet solution. The following controls provided for the neutralization reaction: cell control - wells not infected with virus, negative control sample of mouse serum diluted 1/10, virus control - wells infected with the working dilution of virus diluted two-fold, working virus concentration control - two successive 10-fold dilutions of the working virus concentration were prepared. Neutralizing activity of animal sera was estimated according to serum titer, which registered protection in 50% of wells with cell culture from cytopathic action of virus. The neutralizing antibody titer was calculated using the Reed-Mench method [9].

2.3. Hemagglutination inhibition reaction (HI)

Before HI, obtained serum was treated with RDE (Denka Seiken, Japan) for 18 hours according to the instructions to remove non-specific thermolabile inhibitors and then heated in a water bath at 56°C and inactivate the enzymatic activity of RDE. Sera were examined in HI against 4 hemagglutinating antigen units of the respective virus serotype. To determine the HI titer of inhibition of sera of serotypes H1, B, 0.5% rooster erythrocytes and V-bottom plates were used; for subtype H3 antigen, 1% guinea pig erythrocytes were used. When calculating geometric mean titers (GMT), values in HI <1/10 were considered to be 5. Influenza viruses of strains A/Victoria/2570/2019 (H1N1)pdm09; A/Cambodia/e0826360/2020 (H3N2); B/Washington/02/2019 (B/Victoria); B/Phuket/3073/2013 (B/Yamagata) were used as antigens. Also, HI used complementary ferret antiserum to these strains designed to control the immunogenicity of existing influenza vaccines (CDC, USA) [10].

2.4. Electron microscopy

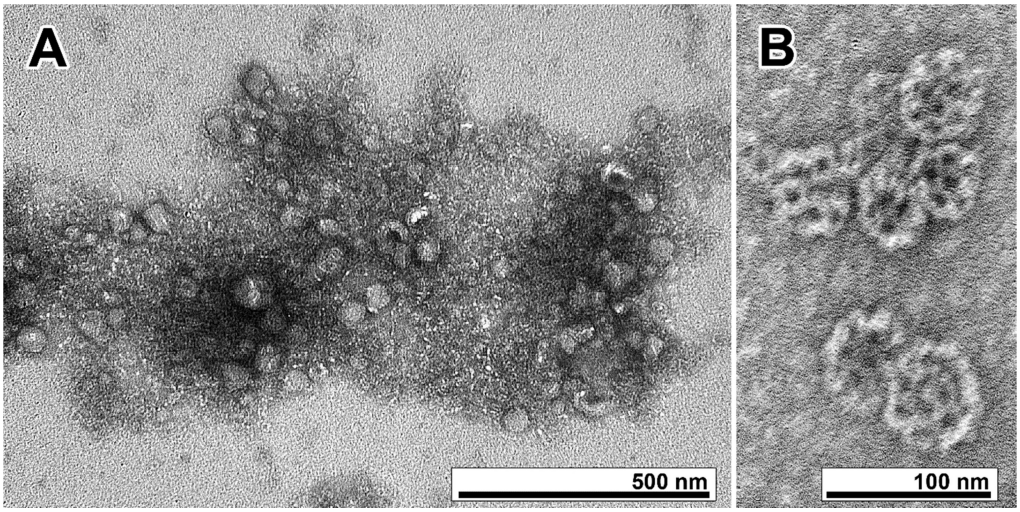
For electron microscopic examination, the obtained preparations, subjected to sterilizing filtration through a 0.22 μm filter nozzle, were applied in a volume of 15 ml to a membrane centrifuge concentrator with a 100 kDa membrane (Millipore). The concentrate centrifuged for 1 h at 1500 g (12150-H rotor, Sigma). The concentrate transferred to 150 μl tubes. Images obtained by negative staining with 1% uranyl acetate with analysis on a JEM-1400 transmission electron microscope (JEOL, USA) [11].

2.5. High performance liquid chromatography

The content of Quillaja soap saponins in ISCOM adjuvant was determined by HPLC on an LC-20 Prominence chromatograph (Shimadzu) with an SPD M20A diode-matrix detector and a Kromasil 300-5-C4 column (4.6 mm id x 150 mm). The volume of the sample to be analyzed is 50 μl. Mobile phase A: 95% deionized water, 5% acetonitrile, 0.1% trifluoroacetic acid. Mobile phase B: 5% deionized water, 95% acetonitrile, 0.1% trifluoroacetic acid. Linear gradient: increase of mobile phase B from 20% to 65% in a time step of 0 min to 30 min followed by holding 65% of B level up to 35 min. The mobile phase rate was 0.5 mL/min, detection was 215 nm. A dry preparation of Saponin from Quillaja Bark pure (PanReac, Code - A2542) was used as a reference sample [12].

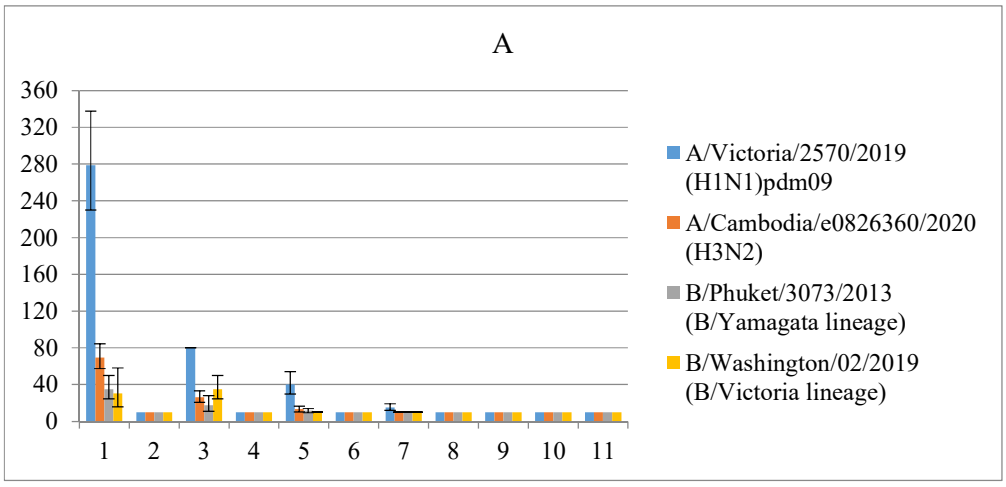
3. RESULTS AND DISCUSSION

A series of ISCOM adjuvant Matrix-V was obtained with a concentration of the active component of *Quillaja saponaria* saponins of 47.9 mg/ml, residual detergent 1.83 mg/ml. The hemolytic titer of this series was 1:2. A single calculated dose of ISCOM adjuvant per animal was 16 μg. The detergent content was below the detection limit of HPLC (less than 10 ng/ml), and no hemolysis was observed. The ultrastructure monitored by electron microscopy. Picture 1 (A) and Picture 1 (B) clearly identify virus-like particles with a characteristic cage-like structure.



Picture 1. Photographs of immunostimulatory complex particles. Photographs of particles of immunostimulatory complexes obtained using *Quillaja saponaria* saponins: A - 100-fold Matrix-V concentrate, B - Ultrastructure of particles of immunostimulatory complexes based on *Quillaja saponaria* saponins

HI results shown in Figure 1. The data obtained in HI indicate that the antigen mixture used is immunogenic without the addition of adjuvant. At the same time, the maximum dose of 1 µg used in this study leads to detectable HI titers only when administered twice; no response was recorded in HI when administered once. The administration of preparations containing Matrix-V adjuvant resulted in a significant increase in the immune response. It should be noted that a protective titer of more than 1:40 to each of the four antigens was formed by a double administration of 10 ng of antigen. This is 750 times lower than the antigen content of the WHO recommended inactivated influenza vaccines.



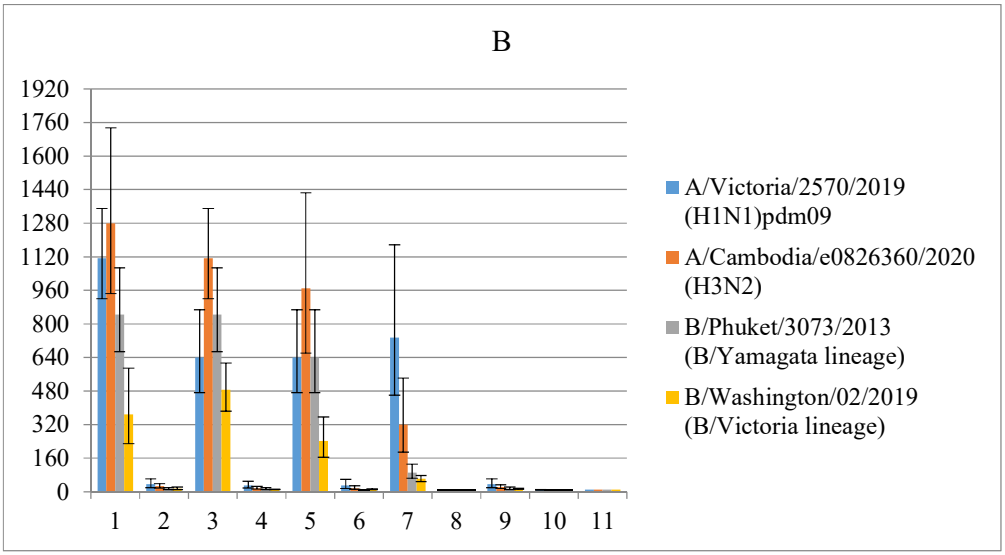


Figure 1. Hemagglutination inhibition titers in blood serum samples of intramuscularly immunized animals taken at 14 (A) and 28 (B) days after immunization. Abscissa axis - names of groups of experimental animals: 1 - Matrix-V + 1 μg antigen; 2 - 1 μg antigen; 3 - Matrix-V + 200 ng antigen; 4 - 200 ng antigen; 5 - Matrix-V + 50 ng antigen; 6 - 50 ng antigen; 7 - Matrix-V + 10 ng antigen; 8 - 10 ng antigen; 9 - Matrix-V + 1 ng antigen; 10 - 1 ng antigen; 11 - negative control Matrix-V without antigen.The ordinate axis is the geometric mean values of inverse antibody titers. The legend indicates the influenza virus antigens used for immunization. Vertical error markers indicate confidence interval values at P=0.95.

The results of double intramuscular immunization with inactivated preparations of Covid-19 (strain hCoV-19/Russia/Moscow171619-031221/2021, (EPI_ISL_8920444), Omicron 1, VA.1) are shown in Figure 2.

Immunization of Balb/c mice with the antigen of strain hCoV-19/Russia/Moscow171619-031221/2021, (EPI_ISL_8920444), Omicron 1, BA.1 was performed with repetition. No significant differences between inverse titers were recorded at P=0.95. At the same time, a significant increase in neutralizing titers compared to the group immunized with Freund's adjuvant complete and antigen-free adjuvant preparation should be noted. The results of this study correlate with the results obtained in the ISCOM study of the adjuvant Matrix-V made using laboratory technology and commercial preparations containing the Matrix-M analogue (Novavax) [4, 13, 14].

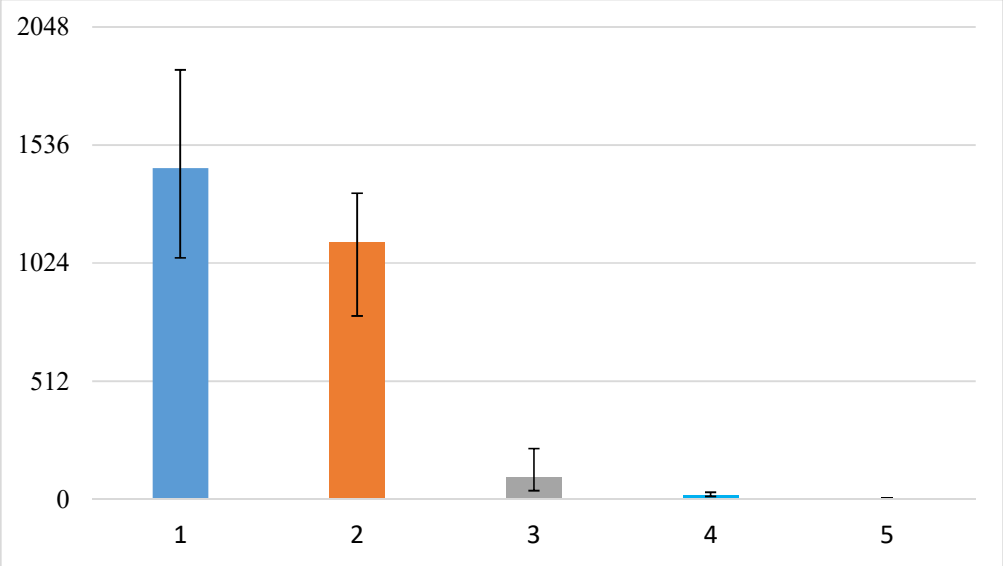


Figure 2. Neutralization reaction data with blood serum samples of doubly intramuscularly immunized animals taken at 21 days after immunization. Abscissa axis - names of groups of experimental animals: 1 - Matrix-V + Omicron; 2 - Matrix-V + Omicron (Repeat); 3 - Freund's adjuvant complete + Omicron; 4 - Omicron; 5 - negative control Matrix-V without antigen. The ordinate axis is geometric mean values of inverse antibody titers. Vertical error markers indicate confidence interval values at P=0.95.

4. CONCLUSIONS

The proposed approach to obtaining ISCOM adjuvant based on *Quillaja saponaria* saponins, quality control by the content of saponins and detergents used in the production allows obtaining a promising adjuvant with the possibility of industrial scaling. On the basis of the data obtained on the immune response, it is possible to conclude that using Matrix-V as an adjuvant, antigen savings of 15-30-fold for drugs administered once and 150-300-fold for drugs administered twice or more can be achieved.

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SUMMARY

A promising approach to immunization is the use of saponin-based virus immunostimulatory complexes (ISCOM) and the creation of antigen complexes (ISCOM-antigen) based on them. Objective: production and study of virus-like immunostimulatory complexes based on *Quillaja saponaria* saponins. Materials and methods: using the tangential filtration method, the preparations of ISCOM adjuvant "Matrix-V", were obtained. Electron microscopic study of the preparations confirmed expected object ultrastructure. The content of saponins, residual detergent in the preparation controlled by HPLC method. Immunization of guinea pigs, Balb/c mice with preparations of ISCOM-antigen (influenza virus A, B; Covid-19) performed intramuscularly. Blood serum samples of immunized animals examined in hemagglutination inhibition reaction, neutralization assay. Results: ISCOM containing *Quillaja saponaria* saponins were successfully qualified. Concentrations of saponins and residual detergent in the obtained preparations determined and were 47.9 mg/ml and 0.63 mg/ml respectively. As a result of a double intramuscular immunization of mice with 50 ng of hemagglutinin of each subtype, GMT in HI in serum was 1:735 - H1N1pdm09; 1:320 - H3N2; 1:91 - B(Yamagata); 1:60 - B(Victoria). No immune response to the vaccine in the same concentration without ISCOM was detected in HI. When immunized with Covid-19 antigens, virus neutralization titer was 1:20, with the Covid-19-ISCOM-adjuvant complex 1:1114 - 1:1436. The presented results indicate the promising use of ISCOM-based preparations in the development of vaccines.

Keywords: *Virus-like immunostimulatory complexes, ISCOM, adjuvant, saponin, vaccine, immunization, influenza, Covid-19, respiratory infection.*

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