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Immunogenicity and Protectivity of the Peptide Vaccine against SARS-CoV-2

Background. *In 2020, the pandemic caused by novel coronavirus infection has become one of the most critical global health challenges during the past century. The lack of a vaccine, as the most effective way to control the novel infection, has prompted the development of a large number of preventive products by the scientific community. We have developed a candidate vaccine (EpiVacCorona) against novel coronavirus infection caused by SARS-CoV-2 that is based on chemically synthesized peptides conjugated to a carrier protein and adsorbed on aluminum hydroxide and studied the specific activity of the developed vaccine.* ***Aims*** *— study of the immunogenicity and protectivity of the peptide candidate vaccine EpiVacCorona.* ***Methods.*** *The work was performed using standard molecular biological, virological and histological methods.* ***Results.*** *It was demonstrated that EpiVacCorona, when administered twice, spaced 14 days apart, to hamsters, ferrets, and non-human primates (african green monkeys, rhesus macaques) at a dose of 260 μg, which is equal to one inoculation dose for humans, induces virus-specific antibodies in 100% of the animals. Experiments in hamsters showed this vaccine to be associated with the dose-dependent immunogenicity. The vaccine was shown to accelerate the elimination of the virus from the upper respiratory tract in ferrets and prevent the development of pneumonia in hamsters and non-human primates following a respiratory challenge with novel coronavirus.* ***Conclusions.*** *The results of a preclinical specific activity study indicate that the use of EpiVacCorona has the potential for human vaccination.*

Keywords*: coronavirus, peptide vaccine, preclinical studies*

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Introduction

Coronaviruses of the *Coronaviridae* family, circulating in the human population and causing acute respiratory infections, periodically cause outbreaks of severe infections, in particular, severe acute respiratory syndrome (caused by SARS-CoV) or Middle East respiratory syndrome (caused by MERS-CoV). Novel coronavirus SARS-CoV-2, a representative of the genus Betacoronavirus, compared to its close relatives SARS-CoV and MERS-CoV, can be transmitted very quickly from human to human, and the lethality associated with this virus is 30 to 40 times higher than that from influenza virus [1]. Given the rate of the infection spread, noted by the World Health Organization, in September 2020 the number of cases may approach 33 million, and the number of victims would exceed 1 (one) million. The pandemic caused in 2019-2020 by the SARS-CoV-2 virus has set an urgent task for the public health of all countries of the world, which is to develop effective therapeutic and preventive products. Currently, over 180 different types of vaccines against novel coronavirus infection are being developed: subunit vaccines, vector replicating vaccines, vector non-replicating vaccines, nucleic acid-based vaccines, inactivated vaccines, live attenuated vaccines, and vaccines based on virus-like particle [2]. The development of various types of vaccines will make it possible to use these products or their combinations to generate the protective immunity in various target populations. Viral surface protein S is a common target for vaccines developed against novel coronavirus. SARS-CoV-2 protein S consists of two subunits. S1 subunit provides binding of the virus to ACE2 receptor located on the target cell membrane. S2 subunit ensures fusion of the virion envelope and the target cell membrane. Blocking receptor binding and cell membrane fusion functions can provide protection against viral infection. However, there was a possibility that using the full-length SARS-CoV-2 S protein might have several caveats, and the most serious one could be antibody-dependent enhancement of infection as it was previously shown for SARS-CoV-1 spike protein by a group of Chinese scientists [3].

Therefore, we considered the alternative approach that provides the development of protective immunity and excludes the possibility of antibody-dependent enhancement of infection. We used the technology of synthetic peptide vaccines which includes *in silico* design of several immunoactive peptide fragments representing cognate viral antigen, chemical synthesis of peptides followed by conjugation to a high-molecular carrier protein. The most effective constructs were selected for inclusion into the candidate peptide vaccine formulation after studying their immunogenicity, antigenic specificity and protectivity in animal models (data not shown). Peptides selection was based on published spatial structures of homologous SARS-CoV-1 spike protein and on genetic sequences data of novel coronavirus SARS-CoV-2 [4, 5]. Epitopes located near the sites vital for the virus were designed using the original computer modeling methods. The design excluded epitopes that could lead to antibody-dependent enhancement of infec-

tion (including peptide S597-603) and peptides with local antigenic similarity to human protein [3, 6-8]. In order to ensure the robustness of the vaccine to possible mutations of the virus, which would entail changes in antigenic properties, epitopes were selected from the most conserved regions of S protein [7, 8]. Synthesized peptides were covalently bound to a carrier protein. N protein of the novel coronavirus was chosen as a carrier since it is well-conserved and contains virus-specific T-cell epitopes and thus is also involved in production of memory T cells. Finally, we have developed a synthetic peptide vaccine against novel SARS-CoV-2 coronavirus based on chemically synthesized peptides. The vaccine (EpiVacCorona) is a suspension for intramuscular administration containing a composition of chemically synthesized peptide immunogens of the S protein of the SARS-CoV-2 coronavirus conjugated to a carrier protein and adsorbed on aluminum hydroxide. The expected advantages of such a subunit vaccine over other vaccine platforms are as follows:

- effectiveness of the vaccine against antigenically variable strains since the vaccine contains conservative SARS-CoV-2 epitopes;
- vaccine safety: unlike most subunit vaccines, a peptide vaccine contains only short regions of the viral protein, which additionally increases its safety, and it makes it possible to use it in immunocompromised individuals, with immunosuppressive conditions;
- ease of production and stability of components allow scaling the production of hundreds of millions of vaccine doses using state-of-the-art technologies of solid-phase peptide synthesis using automatic synthesizers;
- storage and transportation conditions from 2 to 8 ºC make it possible to use existing logistic processes.

Aims: The objective of this work was a study of specific immunogenic and protective activity of the the peptide candidate vaccine against SARS-CoV-2.

Methods

Animals

The study used laboratory animals susceptible to coronavirus, such as non-human primates, syrian hamsters, and ferrets. Non-human primates of two species (Chlorocebus aethiops, 8 males with an initial body weight of 4.8-6.3 kg, aged 5-16 years, and Macaca mulatta, 8 males with a body weight of 3.9-4.8 kg, aged 2.9-3 years) were received from the breeding facility of Research Institute of Medical Primatology, Sochi, Russia. Male and female Syrian hamsters weighing 0.09-0.12 kg were from the breeding facility at OOO KrolInfo, a limited liability company, Russia; male and female ferrets weighing 0.7-1.3 kg were obtained from the breeding facility at FBRI SRC VB VECTOR, Rospotrebnadzor, Koltsovo, Russia.

Duration of the study

May-September 2020

The study of EpiVacCorona immunogenic properties

The study used intact, SARS-CoV-2-seronegative hamsters, ferrets, and non-human primates. Hamsters are highly sensitive to SARS-CoV-2 and provide a good model for study-

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Иммуногенные и протективные свойства пептидной вакцины против SARS-CoV-2

Обоснование. *В 2020 г. пандемия, вызванная новой коронавирусной инфекцией, стала одним из самых серьезных испытаний для глобального здравоохранения за последнее столетие. Отсутствие вакцины как наиболее действенного способа борьбы против новой инфекции обусловило разработку научным сообществом большого количества профилактических препаратов. Нами была разработана кандидатная вакцина (ЭпиВакКорона) против новой коронавирусной инфекции SARS-CoV-2 на основе химически синтезированных пептидов, конъюгированных на белок-носитель и адсорбированных на гидроксид алюминия, и изучена ее специфическая активность.* ***Цель исследования*** *— изучение иммуногенных и протективных свойств кандидатной пептидной вакцины ЭпиВакКорона.* ***Методы.*** *Работа была выполнена при помощи стандартных молекулярно-биологических, вирусологических и гистологических методов.* ***Результаты.*** *Показано, что ЭпиВакКорона при двукратном введении с интервалом в 14 дней хомякам, хорькам и низшим приматам (африканским зеленым мартышкам, макакам-резусам) в дозе 260 мкг, равной одной прививочной дозе для человека, вызывает индукцию вирусспецифических антител у 100% животных. В опытах на хомяках показано, что вакцинный препарат обладает дозозависимой иммуногенностью, вакцина индуцирует ускорение элиминации вируса из верхних дыхательных путей у хорьков и предотвращает развитие пневмонии у хомяков и низших приматов после респираторного заражения новым коронавирусом.* ***Заключение.*** *Результаты доклинического исследования специфической активности свидетельствуют о перспективности использования ЭпиВакКорона для вакцинации людей.*

Ключевые слова*: коронавирус, пептидная вакцина, доклинические исследования*

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ing the infection. After intranasal challenge the virus replicates in upper and lower respiratory tract and affects lungs. This model can be used to assess the vaccine protectivity in pathomorphological and pathohistological studies and in particular could be useful for dose-finding experiments – to determine the dose and regimen that provides protection against pneumonia. Two groups of 10 hamsters each were immunized twice intramuscularly by LUER 3-part syringe with a capacity of 1ml with a needle of 29G (TsSKB-Progress) at a 14-day interval with two different doses of EpiVacCorona containing 86 μg and 260 μg of the active ingredient (0.3 and 1 vaccination dose for humans, respectively). The third group received placebo - 0.9% sodium chloride.

Ferrets are less susceptible to SARS-CoV-2 and they require larger viral doses to develop the disease as compared to hamsters. In ferrets, SARS-CoV-2 replicates mainly in the upper respiratory tract, and typically the infection proceeds without visible clinical manifestations. This fact makes it possible to use the ferret model for vaccine protective effect against intranasal infection with assessing viral load in nasal swabs of the animals at different time points after challenge. In particular, such a model can be used to examine variations in protective properties of different vaccine batches. Three groups of ferrets, 6 animals each, were immunized twice intramuscularly by LUER 3-part syringe with a capacity of 1ml with a needle of 29G (TsSKB-Progress) with three EpiVacCorona batches at a dose of 260 μg of active ingredient (1 vaccination dose for humans), at an interval of 14 days. The fourth group of animals received placebo — 0.9% sodium chloride.

Nonhuman primates are the closest animal model to humans. The most common primates used in research are the African green monkeys Chlorocebus aethiops and the rhesus monkey Macaca mulatta. However, these two species are known to differ in both their immune reactions and resistance to infections. Therefore, we were interested not only to study the immunogenicity and protectivity of EpiVacCorona vaccine in primates but also to compare the immune responses and susceptibility to coronavirus infection of these two species [9]. Non-human primates 5 animals of each species, were immunized twice intramuscularly by LUER 3-part syringe with a capacity of 1ml with a needle of 29G (TsSKB-Progress) with the EpiVacCorona vaccine at a dose of 260 μg of active ingredient (1 vaccination dose for humans), at an interval of 14 days. A total of 3 primates of each species received placebo — 0.9% sodium chloride.

After 14 and 28 days post first immunization serum antibodies to SARS-CoV-2 antigens were measured using ELISA and viral neutralization assays.

The study of EpiVacCorona protective properties

All studies to assess protective properties of the vaccine were carried out in a maximum-containment laboratory of FBRI SRC VB VECTOR, Rospotrebnadzor in compliance with the requirements for safe work with microorganisms of I-II pathogenicity groups. SARS-CoV-2 virus, strain nCoV/Victoria/1/2020, propagated in Vero E6 cells with infectivity 107 focus forming units/ml (FFU/ml) was used. Hamsters and ferrets were intranasally challenged with 102 and 103 FFU, respectively, of SARS-CoV-2 virus 28-30 days after the first administration of EpiVacCorona. During 14 days after the challenge, the animals were observed for clinical signs, and viral load in nasal washes was measured. Primates were intranasally challenged with 106 TCID50 of the SARS-CoV-2 nCov/Victoria/1/2020 strain 30 days after the first vaccine administration. During 14 days after the challenge, the animals were observed for clinical signs, and viral load in the nasal washes was measured.

Cell culture-based measurement of SARS-CoV-2 viral load in biological samples

Nasal washes of laboratory animals were used to prepare 10-fold dilutions inoculated in Vero E6 cell monolayers in 96-well plates. The growth medium was removed from the wells of a flat-bottomed plate with monolayer; the monolayer was washed twice using maintenance nutrient medium (DMEM, 100 U/ml benzylpenicillin, 100 μg/ml streptomycin, 300 μg/ml L-glutamine, 2% fetal bovine serum). Dilutions of samples under study were added and incubated for 1 hour at 37 °C and 5% CO_2, with medium removal from wells and a single wash of a plate with the maintenance medium. A total of 150 μl of the maintenance medium was added to each well of the plate. After 18-20 hours of incubation at 37 °C and 5% CO_2, the medium was removed from wells, 100 μl of 80% acetone (cooled to minus 20 °C) was added. Following a 10-15-min incubation, acetone was removed, and the plate was washed with phosphate buffered saline (PBS). A total of 100 μl of diluted human monoclonal antibodies to SARS-CoV-2 (Anti-N protein SARS-CoV-2 mAb_IgG, "SanyouBiopharmaceuticals", antibodies to PBS ratio 1:2000) was added to each well. The plate was incubated at 37 °C for 1 hour, the wells were washed with PBS four times, and secondary rabbit antibodies to human IgG conjugated to horseradish peroxidase (Abcam) were added at a dilution of 1:1000. After 30 minutes of incubation, the wells were washed four times with PBS, and AEC (3-amino-9-ethylcarbazole, Sigma) solution was added. After 30 min of incubation, the solution was removed, the plate was washed once with PBS, the infected cells stained in red-brown were counted using an inverted microscope, and viral titer was determined by the number of focus-forming units per 1 ml of wash (FFU/ml).

Quantification of SARS-CoV-2 viral load in biological samples by RT-PCR

Isolation of RNA from nasal washes was performed using a "Ribo-sorb" reagent kit (CRIE, Russia) according to the manufacturer's instructions. The synthesis of cDNA from isolated RNA was carried out using a "Reverta-L" (CRIE, Russia) reagent kit for reverse transcription reaction in accordance with the manufacturer's instructions. Amplification of SARS-CoV-2 cDNA fragments, synthesized on SARS-CoV-2 RNA template in reverse transcription reaction, was carried out using Vector-PCR_{RT}-COVID-19-RG reagent kit (FBRI SRC VB VECTOR, Russia) according to the manufacturer's instructions. The test results are interpreted as described in the kit's instruction.

Identification of virus-specific antibodies using ELISA

Specific antibodies were identified using inactivated SARS-CoV-2 virus, strain nCoV/Victoria/1/2020, propagated in Vero E6 cells. An immunosorbent, which is a 96-well polystyrene plate of high sorption capacity containing an immobilized inactivated natural SARS-CoV-2 antigen. Inactivation of the virus was performed by adding beta-propiolactone to antigens purified by precipitation at a final concentration of 0.5%. The incubation is carried out for 2 hours at 4 °C and stirring. After that, beta-propiolactone is decomposed by heating for 2 hours at 37 °C. For titration, two-fold dilutions of the sera of animals immunized with the vaccine and control animals (not immunized) are prepared in the range of 1:40-1:2560. After incubation for 30 min at 37 °C, plate wells were washed five times with PBST containing Tween-20. After washing, 100 μl of the solution of Staphylococcus aureus protein A conjugated

to horseradish peroxidase at a final concentration of 1.26 μg/ml (Biosan, Novosibirsk) was added to the wells. After a 30-minute incubation at 37 °C, the wells were washed and 0.05% tetramethylenebenzidine solution was added. The optical density of the solutions was measured at a wavelength of 450 nm. The antibody titer is the maximum dilution of studied serum, at which the optical density of the solution exceeds the average optical density of the negative control.

Detection of virus-neutralizing antibodies in Vero cell culture

The studied sera were thermally inactivated for 30 min at 56 °C prior to virus neutralization assay. Sterile round-bottom plates were used to prepare serial two-fold dilutions of the studied sera in a maintenance nutrient medium (DMEM, 100 U/ml benzylpenicillin, 100 μg/ml streptomycin, 300 μg/ml L-glutamine, 2% fetal bovine serum (Invitrogen). The viral suspension containing 200 FFU of SARS-CoV-2 was added to each well of the plate, except cell controls, with subsequent incubation for 1 h at 37 °C. A flat-bottomed 96-well plate with Vero E6 monolayer of more than 90% confluence was then used. The growth medium was removed from the wells of a flat-bottomed plate with a monolayer of Vero cells, and the monolayer was washed twice with the maintenance medium. After a 60-minute incubation, 100 μl was transferred from a round-bottom plate with serum dilutions to the corresponding wells of the plate with the cell monolayer. After incubation for 1 hour at 37 °C and 5% CO_2, the medium was removed from the wells and the plate was washed once with the maintenance medium. The 150-μl volume of the maintenance medium was added to all wells of the plate. After that, FFUs were stained. After 18-20 hours of incubation at 37 °C and 5% CO_2, the medium was removed from the wells, 100 μl of 80% acetone (cooled to minus 20 °C) was added. After a 10-15-min incubation, acetone was removed, and the plate was washed with phosphate buffered saline (PBS). A total of 100 μl of diluted human monoclonal antibodies to SARS-CoV-2 (Anti-N protein SARS-CoV-2 mAb_IgG, "SanyouBiopharmaceuticals", antibodies to PBS ratio 1:2000) was added to each well. Protein N is expressed in amounts much larger than that of protein S and this fact Anti-N antibodies are widely propagated in coronavirus infected cells that allows to visualize infected cells with high sensitivity with anti-N protein antibodies. The plate was incubated at 37 °C for 1 hour, the wells were washed with PBS four times, and secondary rabbit antibodies to human IgG conjugated to horseradish peroxidase (Abcam) were added at a dilution of 1:1000. After 30 minutes of incubation, the wells were washed four times with PBS, and AEC (3-amino-9-ethylcarbazole, Sigma) solution was added. After 30 min of incubation, the solution was removed, the plate was washed once with PBS, the infected cells stained in red-brown were counted using an inverted microscope, and viral titer was determined by the number of focus-forming units.

The titer of the neutralizing serum antibodies is a dilution at which the amount of FFU is reduced by 50% compared to the average value of FFU in control wells.

Hystological studies

Histological examination was performed for lung samples taken from hamsters and primates 6-7 days after intranasal viral infection (SARS-CoV-2, strain nCoV / Victoria / 1/2020). The samples were fixed in a 10% solution of neutral formalin for histological studies (BioVitrum, Russia) for 48 h. The obtained material was processed further according to the standard technique including sequential dehydration in alcohols of increasing concentration, soaking in a xylene-paraffin mixture, and embedding into paraffin blocks. Paraffin sections 4–5 μm thick were prepared using an automatic rotary microtome NM-360 (Germany). Sections were stained with hematoxylin and eosin. Light-optical examination and microphotography were performed on an AxioImager Z1 microscope (Zeiss, Germany) using the AxioVision 4.8.2 software package (Zeiss, Germany).

X-ray studies

X-rays of thoracic cavity organs were taken in animals using the X-ray device, GIERTH HFX90V (Germany / Japan), with a SCOPE 801CW Ultraleicht digital radiography system with a wireless flat-panel detector (27.4 x 35 cm) CDXI-801CW and CANON NE Control-Software. The X-ray diffraction patterns were taken at an irradiation power of 52 kV and an exposure of 0.06 mAs / sec. The radiography was performed in primates anesthetized with the intramuscular injection of Zoletil 100 (Virbac) and Xyla (Holland) at doses of 10 mg / kg and 10 mg / animal, respectively.

Ethical review

All procedures involving animals planned within the preclinical study were reviewed and approved by the Bioethics Committee of FBRI SRC VB VECTOR, Rospotrebnadzor (application: SRC VB VECTOR/02-05.2020).

Statistical analysis

The analysis of the dynamics of the studied indicators within the groups under study was carried out using Friedman test and Nemenyi post-hoc test. Statistical analysis of paired samples was carried out using Wilcoxon test. Analysis of unpaired samples was carried out using Kruskal-Wallis test and Dunn's post-hoc test. Pairwise comparisons of unrelated samples were also performed using Mann-Whitney test. Correction for multiple testing in all cases was carried out using the Bonferroni method. Frequency analysis was performed using Fisher's exact test (when comparing 2 groups) or Fisher-Freeman-Halton test (when comparing 3 or more groups). Statistical analysis and plotting were performed using specialized software environment for computing and statistical analysis R [10-12].

Results

Vaccine design

The peptide vaccine comprising short immunogenic peptides conjugated on a carrier protein elicits potent and targeted immune responses to only several B-cell epitopes that are located in functionally important sites of viral proteins. To design the vaccine, we used data of X-ray diffraction analysis of coronavirus proteins and data on the genetic sequences encoding the proteins of novel coronavirus, which were retrieved from the GISAID database [4, 5]. Linear B-cell epitopes in S protein located near sites vital for the virus were designed using computer simulation methods. When designing, we excluded epitopes that could lead to an antibody-dependent enhancement of infection or those that have antigenic similarity to human proteins and could cause an immunopathological reaction [6-8]. To ensure the vaccine's resistance to the possible mutations of the virus, which would lead to a change in antigenic properties, epitopes were selected from the most conserved regions of the S protein [7, 8]. A total of 7 peptides carrying linear B-cell epitopes of S protein were designed and chemically synthesized in the form of peptides of 20-31 amino acid residues, which were then conjugated to a chimeric recombinant carrier protein. Novel SARS-CoV-2 N protein was chosen as a carrier protein that

enhances the immunogenicity of the peptides. This protein is conserved, induces no virus neutralizing antibodies, but contains virus-specific T-cell epitopes and is involved in memory T-cell production. NP-specific T cells could be detected in more patients than those for other viral proteins, and the numbers of IFN-γ-secreting NP-specific T cells were much higher than those of S-RBD [13]. These 7 active substances of the candidate vaccines were adsorbed separately on aluminum hydroxide (Figure 1). Such a vaccine does not contain viral proteins or elements of the viral genome; therefore, instead of many dozens and hundreds of epitopes, whose presence is typical of conventional vaccines, the synthetic peptide vaccine contains only a few epitopes that generate a focused protective immune response. A total of 7 groups of ferrets were separately injected twice intramuscularly with seven candidate vaccines to assess the immunogenicity and protectivity of each construct against novel coronavirus. Three most immunogenic and protective peptide conjugates were selected and mixed in ratio 1:1:1 with subsequent adsorption on aluminum hydroxide to produce EpiVacCorona epitope peptide vaccine [14].

Immunogenicity of the EpiVacCorona vaccine

At the first stage of the work, the immunogenic properties of the EpiVacCorona vaccine were studied in laboratory animals (hamsters, ferrets, non-human primates). All animals received vaccine injections twice, spaced 14 days between the injections; the route of administration was intramuscular injections.

Figure 2 shows the results of the immunogenicity study of the EpiVacCorona vaccine in hamsters. On day 14 following the second immunization of hamsters with two different doses of 260 μg and 86 μg, the vaccine induces an immune response in 100% of hamsters to the vaccine antigens. A 3-fold decrease in the vaccine dose leads to a dose-dependent decrease in the geometric mean titer (GMT) to the vaccine antigens. On day 14 following the second vaccination, the GMT in the group of hamsters that received the full dose of the vaccine was 1: 11943; in the group that received 1/3 of the dose, the GMT was 1: 6400 in relation to the vaccine antigen. When using the whole virion antigen of the SARS-CoV-2 coronavirus, the GMT was 1: 4525 and 1: 1600 for vaccine doses of 260 μg and 86 μg, respectively.

Following the immunization of ferrets with three batches of the EpiVacCorona vaccine at a dose of 260 μg, 17 out of 18 (94%) animals developed specific antibodies with titers from 1: 800 to 1: 6400, on day 14 following the first vaccination. On day 14 following the second vaccination, 100% of ferrets developed high levels of specific antibodies, and the GMT in three groups of ferrets immunized with three batches of the EpiVacCorona vaccine was 1: 9051 to 1: 10159 (Figure 2). The EpiVacCorona vaccine was demonstrated to induce antibodies in high titers in 100% of primates to the vaccine antigens and to the whole virion antigen of the SARS-CoV-2 coronavirus, on day 14 following the second vaccination.

In green monkeys, the GMT to the vaccine antigen was 1: 11143, and it was 1: 10159 to the whole virion antigens of the SARS-CoV-2 coronavirus, while in rhesus macaques during these periods the GMT to the vaccine antigen was 1: 12800, and it was 1: 7352 to the whole virion antigens. In 100% of green monkeys, the neutralizing antibodies were found in a titer of 1:40, on day 14 following the first vaccination.

Protectivity of the EpiVacCorona vaccine

On day 14 following the second vaccination, all immunized animals were intranasally challenged with the novel SARS-CoV-2 coronavirus at a dose of 10^2 focus-forming units (FFU) for hamsters, at a dose of 10^3 FFU for ferrets, and at a dose of 10^6 TCID50 for primates. The hamsters were euthanized on days 6 and 14 after challenge, their lung / body mass index was measured, and a histological examination of the lungs was performed. On day 6 after challenge, the lung / body mass index statistically significantly differs in the placebo group (0.0108 ± 0.0004 – the mean value and the standard error) from those of groups vaccinated with EpiVacCorona at a dose of 260 μg (0.0080 ± 0.0023) and at a dose of 86 μg (0.0086 ± 0.0008) (Figure 4).

In the histological examination of lung sections, severe pathological changes were observed in the placebo group of hamsters where there was a complete loss of the epithelial lining

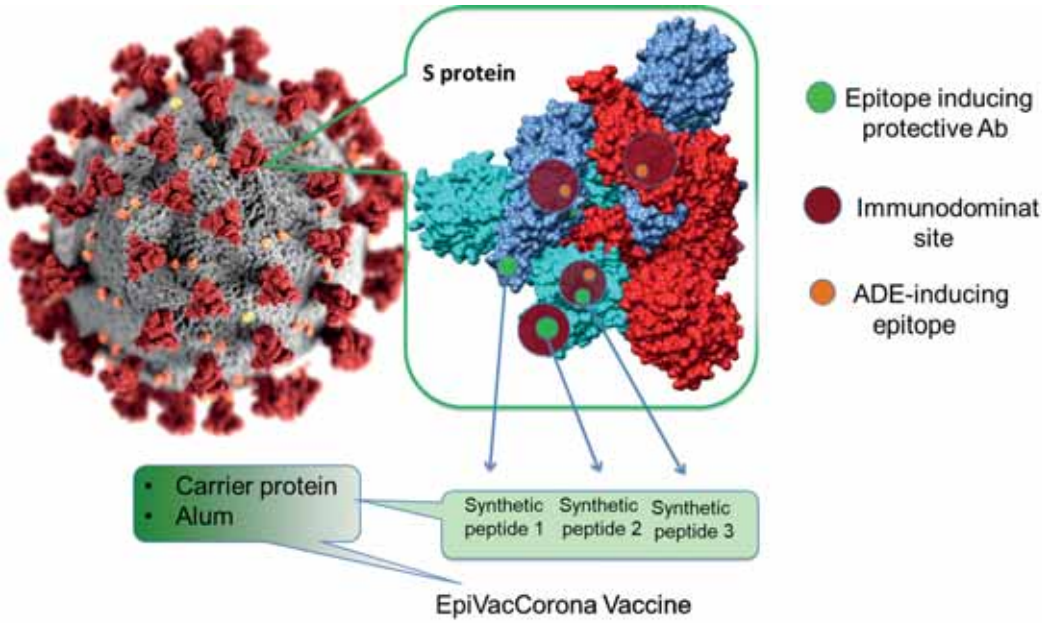

Figure 1. Schematic illustration of S protein epitope locations. Vinous color shows the locations of coronavirus S protein immunodominant sites, orange color shows the epitopes that cause antibody-dependent enhancement of infection, green color shows epitopes included in the EpiVacCorona vaccine. This illustration uses materials from the study [10], Protein Data Base 6ACJ.pdb and CDC Image Library PHIL ID # 23312.

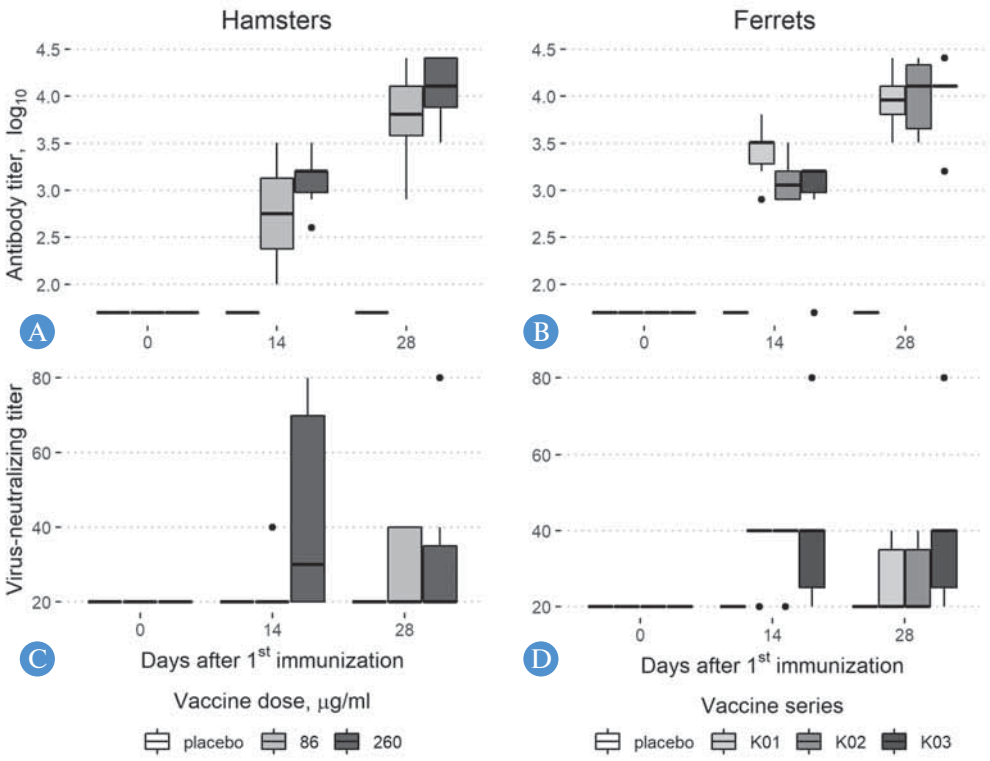

Figure 2. Findings on EpiVacCorona immunogenicity in hamster and ferret experiments. All animals were immunized twice 14 days apart; the route of administration was intramuscular. **A**: Serum antibody titers of hamsters before EpiVacCorona administration, and 14 and 28 days after the first immunization. **B**: Serum antibody titers of ferrets before EpiVacCorona administration, and 14 and 28 days after the first immunization. **C**: Titers of virus-neutralizing antibodies in the sera of hamsters before EpiVacCorona administration, and 14 and 28 days after the first immunization. Two vaccine doses were studied: 260 and 86 μg. **D**: Titers of virus-neutralizing antibodies in the sera of ferrets before EpiVacCorona administration, and 14 and 28 days after the first immunization. Three batches of the vaccine at a dose of 260 μg were studied.

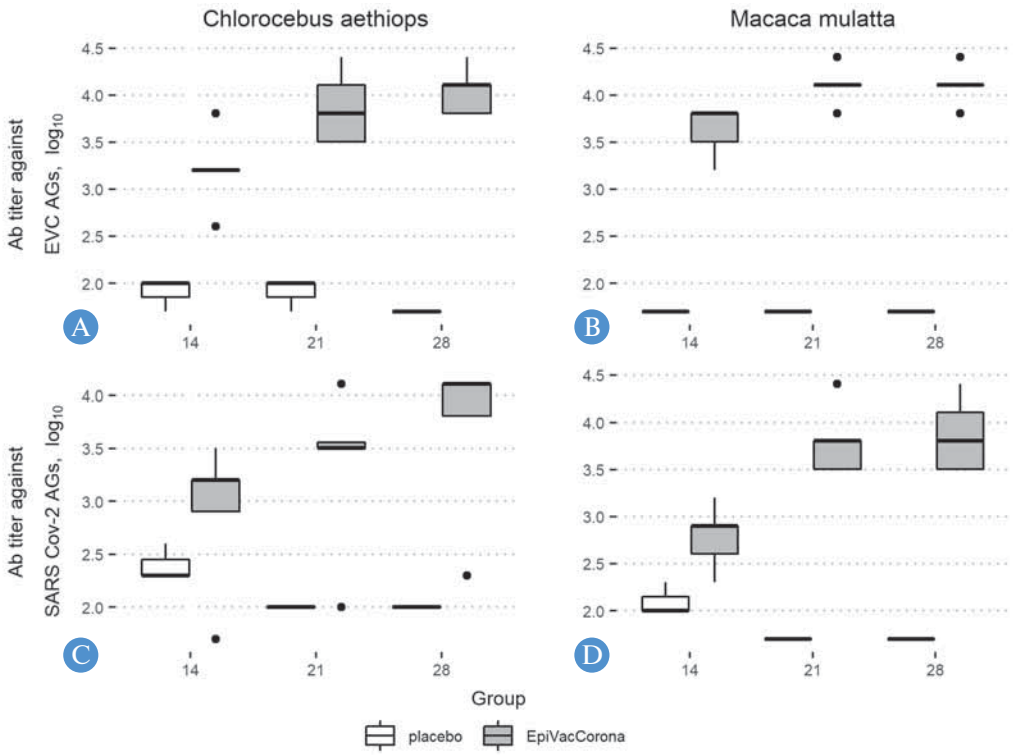

Figure 3. Immune responses to coronavirus antigens in EpiVacCorona-vaccinated green monkeys and rhesus macaques. All animals of EpiVacCorona group were immunized twice intramuscularly 14 days apart at a dose of 260 μg. **A**: Titers of serum antibodies to EpiVacCorona antigen in primates *Chlorocebus aethiops* 14, 21 and 28 days after the first immunization. **B**: Titers of serum antibodies to EpiVacCorona antigen in primates *Macaca mulatta* 14, 21 and 28 days after the first immunization. **C**: Titers of serum antibodies to inactivated SARS-CoV-2 antigens in primates *Chlorocebus aethiops* 14, 21 and 28 days after the first immunization. **D**: Titers of serum antibodies to inactivated SARS-CoV-2 antigens in primates *Macaca mulatta* 14, 21 and 28 days after the first immunization.

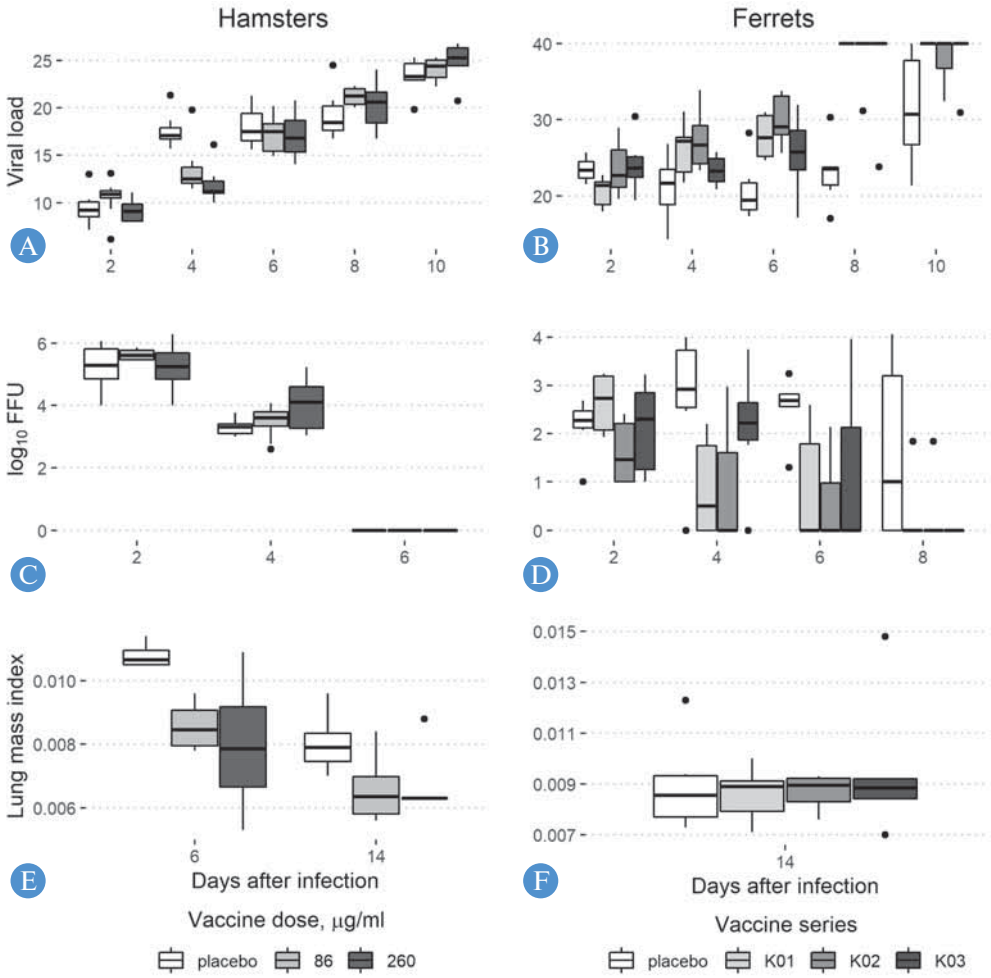

Figure 4. EpiVacCorona protectivity in hamsters and ferrets following coronavirus challenge. All animals were immunized twice intramuscularly 14 days apart. **A**: Viral load, as measured by Ct value, im hamster nasal washes on days 2, 4, 6, 8 and 10 after intranasal coronavirus challenge. Two vaccine doses were studied: 260 and 86 μg. **B**: Viral load, as measured by Ct value, im ferret nasal washes on days 2, 4, 6, 8 and 10 after intranasal coronavirus challenge. Three vaccine batches at a dose of 260 μg were studied. **C**: Viral load, as measured by the number of FFU, in nasal washes of hamsters on days 2, 4, 6, 8 and 10 after intranasal coronavirus challenge. Two vaccine doses were studied: 260 and 86 μg. **D**: Viral load, as measured by the number of FFU, in nasal washes of ferrets on days 2, 4, 6, 8 and 10 after intranasal coronavirus challenge. Three vaccine batches at a dose of 260 μg were studied. **E**: Lung/body weight index measured on days 6 and 14 after intranasal coronavirus challenge of hamsters. Two vaccine doses were studied: 260 and 86 μg. **F**: Lung/body weight index measured on days 6 and 14 after intranasal coronavirus challenge of ferrets. Three vaccine batches at a dose of 260 μg were studied.

of small bronchi and bronchioles, there were foci of necrotization, plasmorrhagia of the vascular walls, and sizable atelectasis zones. In animals vaccinated with a maximum dose of 260 μg of the EpiVacCorona vaccine, only local phenomena of edema and inflammatory cell infiltration were observed, dystrophic changes in the epithelium of small bronchi and blood vessels of the microvasculature were observed in small areas of the lung parenchyma (Figure 5).

For ferrets, it was shown that on day 6 after challenge, the viral RNA load in the groups of vaccinated animals was significantly reduced (more than 100 times) compared to that of the placebo group. Coronavirus from nasal washings was detected in 6 out of 18 vaccinated ferrets (33%) and in 100% of animals in the placebo group. On day 8 after challenge, viral RNA is isolated in the nasal washings in 11% of the vaccinated ferrets and in 67% of animals in the placebo group while the virus shows itself in cell culture in 50% of animals in the placebo group and in 11% of the vaccinated animals. The time for virus elimination from the upper respiratory tract in the vaccinated ferrets is reduced by more than 6 days compared to the placebo group (Figure 4).

For primates, it was shown that on days 2, 4, 6, 8, 10, and 12 after challenge no statistically significant differences in the viral loads in the nasal swabs were recorded for vaccinated and placebo groups, within each of the two species. The rate of elimination of coronavirus from the surface of the nasal mucosa in green monkeys was higher than that in rhesus macaques in both the vaccinated and placebo groups. The body weight of primates after challenge of the vaccinated and placebo groups did not change significantly. However, in primates of each species, *Chlorocebus aethiops* and *Macaca mulatta*, in the control groups, after challenge, body temperature was recorded, which was by 1 °C higher than that in the vaccinated animals (Figure 6).

On day 14 after viral challenge the primates were examined with X-ray roentgenography for the signs of viral pneumonia. At the same time, in the placebo group, after challenge with the SARS-CoV-2 coronavirus, 67% of primates of each species, *Chlorocebus aethiops* and *Macaca mulatta*, had radiological signs of viral pneumonia (Figures 7, 8).

There are two main histological signs of lung damage in coronavirus disease — diffuse alveolar damage and al-

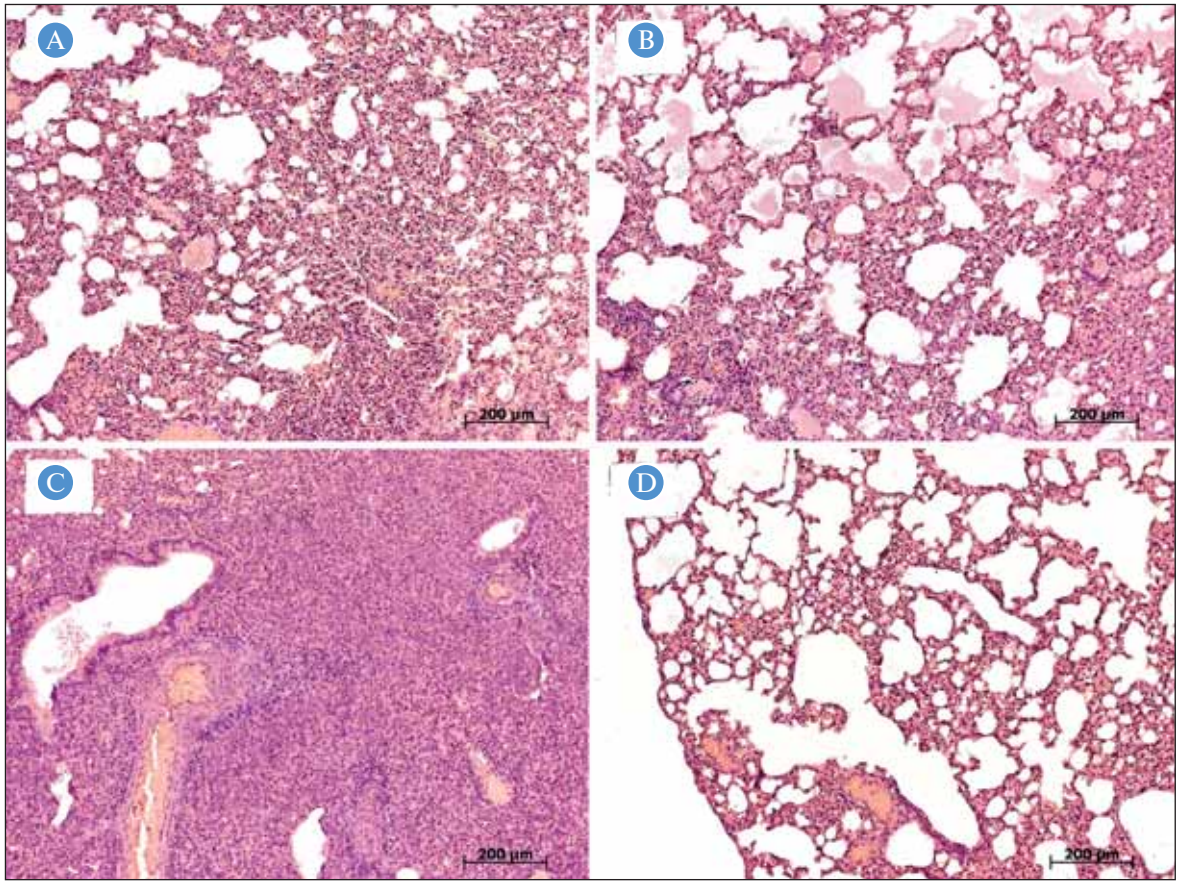

Figure 5. Histological studies of the lungs of hamsters infected with SARS-CoV-2. A. Lungs of hamsters immunized with EpiVacCorona at a dose 260 μg 6 days after coronavirus challenge. Atelectasis, plasmorrhages, pronounced local inflammatory cell infiltration by lymphoid cells and neutrophilic granulocytes are observed. Small bronchi without epithelium, but diapedesis is either weak (lymphocytes) or barely noticeable.
B. Lungs of hamsters immunized with EpiVacCorona at a dose 86 μg 6 days after coronavirus challenge. Severe atelectasis, widespread neutrophil infiltration, vasculitis and destruction of individual small bronchi, small loci of necrotization, alveolar cavities are filled with plasma and erythrocytes.
C. Lungs of unvaccinated hamsters 6 days after coronavirus challenge. The parenchyma is practically airless due to alveolar hemorrhagic syndrome and pronounced infiltration by mononuclear cells. Microangiopathy, plasmorrhagia of vascular walls, interalveolar septa are sharply thickened, bronchiolitis and hyaline membranes.
D. Lungs of unvaccinated hamsters before coronavirus challenge.

veolar hemorrhagic syndrome. Both signs are observed in the animals within the experiment with much higher intensity in controls compared with the vaccinated primates. The targets of the primary damage are type 1 and type 2 alveolar pneumocytes and capillary endothelium (the target is not for the virus). Hemorrhagic syndrome is caused by total damage to the capillaries of alveolar septa, which results in pulmonary edema (high permeability pulmonary edema), arterial hypoxia and respiratory failure. Diffuse alveolar damage is a stagewise process. Damage to aerohematic barrier leads to intra-alveolar edema and fibrin exudation followed by hyaline membrane formation, which prevents gas exchange (exudative phase). Subsequently, intraalveolar fibrosis occurs with the organization (sclerosing) of the affected tissue (proliferative phase). Pathological findings in all vaccinated animals corresponded to the initial stage of proliferation phase. However, controls demonstrated lung changes characteristic of the proliferative phase along with signs characteristic of the early (exudative) phase, which may indicate a continuing pathological effect (Figures 9A — 9D).

Our results demonstrate that the area of severe damage in green monkeys is smaller (about 25%) than in macaques (40-45%). Alveolar hemorrhagic syndrome was, however, very pronounced in green monkeys, especially in controls. Notably, massive perivascular, intrabronchiolar and intraalveolar hemorrhages and hemorrhagic infarctions are signs of the early phase of the pathological process. This is a very significant difference between the two primate models, and it was observed both macroscopically and histologically.

Discussion

A vaccine has the potential to establish herd immunity to reduce morbidity, prevent the spread of the virus and reduce the social and economic burden of COVID-19. Although the experimental vaccine based on any technological platform need to meet safety requirements, every type of drug may have specific limitations in its use among certain populations (children, pregnant women, people with immunosuppressive conditions). Among all the developed types of vaccines (live attenuated, based on viral vectors, subunit, etc.), vaccines based on peptide antigens are one of the safest. This is due to the fact that epitopes that cause antibody-dependent enhancement of infection are excluded at the stage of vaccine design, and reactogenic or allergenic effects are minimized. Low immunogenicity might be the disadvantage of that type of the vaccine, but the problem is solved using the adjuvants [15].

The developed EpiVacCorona vaccine, when administered twice at an interval of 14 days to hamsters, ferrets and non-

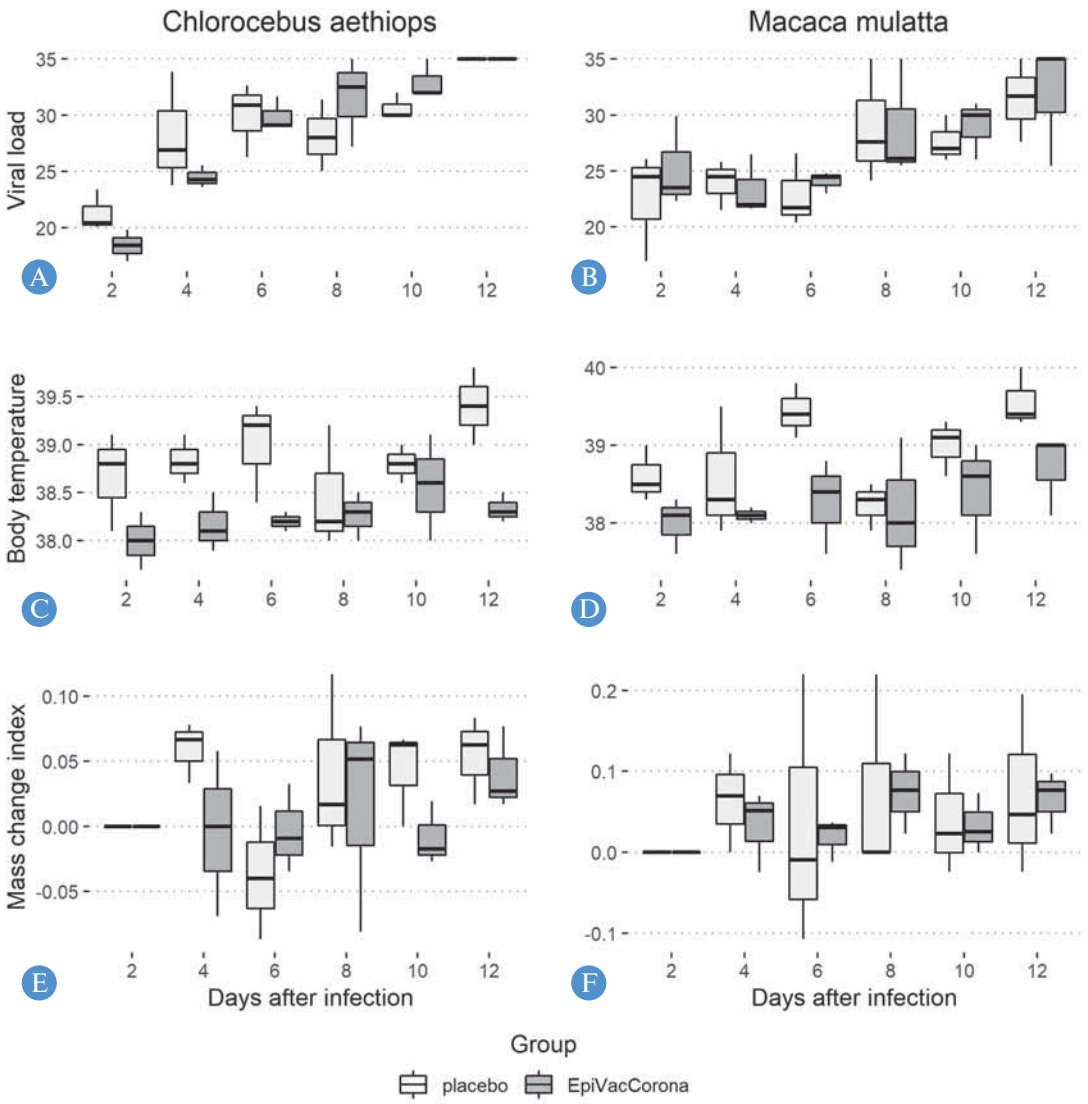

Figure 6. EpiVacCorona protectivity in green monkeys and rhesus macaques as measured by viral load in URT, body temperature and weight loss following coronavirus challenge. All animals of EpiVacCorona group were immunized twice intramuscularly 14 days apart at a dose of 260 μg. **A**: Viral load, as measured by Ct value, in *Chlorocebus aethiops* nasal washes on days 2, 4, 6, 8, 10 and 12 after intranasal coronavirus challenge. **B**: Viral load, as measured by Ct value, in *Macaca Mulatta* nasal washes on days 2, 4, 6, 8, 10 and 12 after intranasal coronavirus challenge. **C**: Body temperature of primates *Chlorocebus aethiops* on days 2, 4, 6, 8, 10 and 12 after intranasal coronavirus challenge. **D**: Body temperature of primates *Macaca Mulatta* on days 2, 4, 6, 8, 10 and 12 after intranasal coronavirus challenge. **E**: Body weight change in primates *Chlorocebus aethiops* on days 2, 4, 6, 8, 10 and 12 after intranasal coronavirus challenge. **F**: Body weight change in primates *Macaca Mulatta* on days 2, 4, 6, 8, 10 and 12 after intranasal coronavirus challenge.

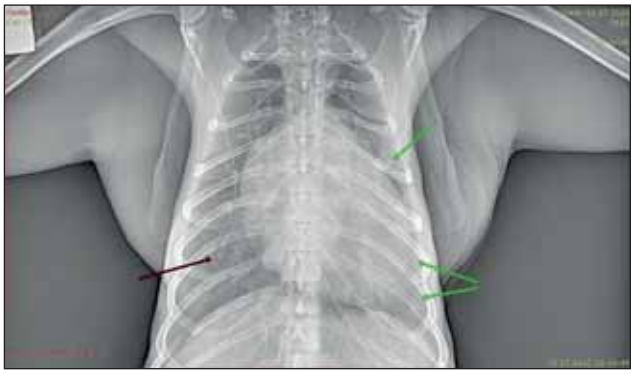

Figure 7. Anterior X-ray of African green monkey thoracic organs 14 days after virus challenge. Unvaccinated animal. Arrows indicate pulmonary consolidation of uneven intensity in lower and middle areas on the left, enhanced pulmonary pattern in lower areas on the right.

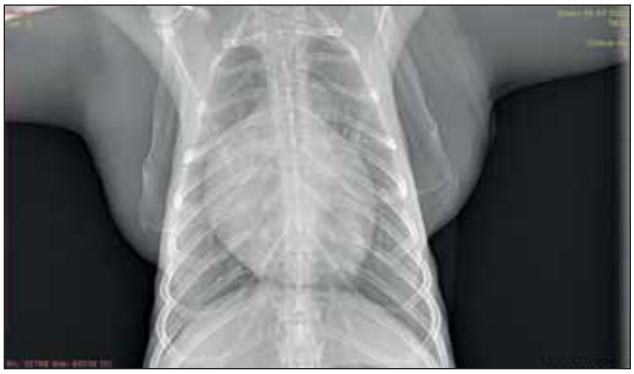

Figure 8. Anterior X-ray of African green monkey thoracic organs 14 days after virus challenge. Vaccinated animal. No signs of pneumonia.

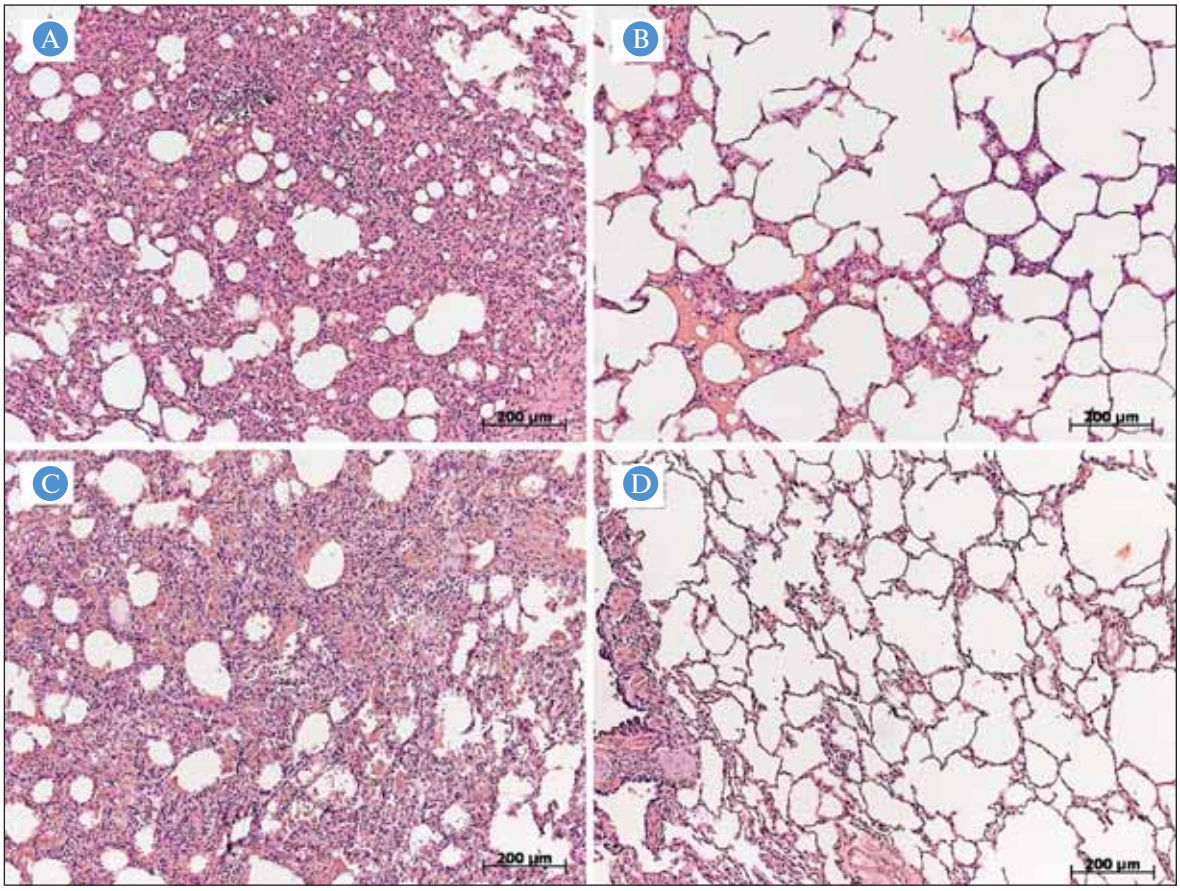

Figure 9. Histological studies of the lungs of primates infected with SARS-CoV-2. A. Primate *Clorocebus aethiops* # 9618 - placebo. Dense atelectasis, pronounced edema and inflammatory infiltration by lymphoid cells and neutrophils. Loss of airiness is accompanied by spasm of blood vessels and small bronchi. Stasis of erythrocytes in the vessels of the microvasculature. Solitary vasculitis and bronchiolitis. Staining with hematoxylin and eosin. The bar is shown in the picture. B. Primate *Clorocebus aethiops* # 9628 - placebo. Vascular hyperemia, hemorrhages and plasmorrhages in the alveolar cavity. Thrombosis of small arterial-type vessels. Plasmorrhagia of blood vessel walls, solitary small foci of fibrinoid necrosis. Staining with hematoxylin and eosin. The bar is shown in the picture. C. Primate *Macaca mulatta* # 9643 - EpiVacCorona vaccine. A small focus of plasmorrhagia and hemorrhage in the alveolar cavity, moderate lymphocytic infiltration and edema of the interalveolar septa. Perifocal compensatory emphysema. Staining with hematoxylin and eosin. The bar is shown in the picture. D. Primate Clorocebus aethiops # 9616 - EpiVacCorona vaccine. Small peribronchial accumulations of lymphocytes are observed. Moderately pronounced emphysematous changes in lung tissue. Staining with hematoxylin and eosin. The bar is shown in the picture.

human primates (African green monkeys, rhesus macaques) at a dose equal to one vaccination dose for humans, induces virus-specific antibodies in 100% of animals. Studies on hamsters demonstrated dose-dependent immunogenicity of the vaccine. The vaccine was shown to accelerate the elimination of the virus from upper respiratory tract (ferrets) and prevent the development of pneumonia in laboratory animals (hamsters, primates) following a respiratory challenge with novel coronavirus. X-ray studies of the lungs of hamsters and primates following coronavirus challenge confirmed the complete absence of pneumonia signs in vaccinated animals of both species. Histological studies of hamster and primate lung tissues also showed a significant decrease in the degree of lung damage in vaccinated animals after intranasal challenge with the new coronavirus.

EpiVacCorona induces a robust immune response to the peptide vaccine antigens with GMT value of 1:11943 for antibodies to the vaccine antigen in sera of hamsters vaccinated with a dose of 260 µg 2 weeks after the second vaccination. After the same period, 100% of vaccinated ferrets have developed a high level of specific antibodies with geometric mean titers ranging from 1:9051 to 1:10159 in three groups of ferrets immunized with three batches of EpiVacCorona. In primates, the vaccine induces high antibody titers to the vaccine antigens and the whole-virion antigen of the coronavirus in 100% of animals 2-3 weeks after the first vaccination. After 4 weeks post first vaccination, GMTs of antibodies to the vaccine antigen in rhesus macaques and African green monkeys reach the values of 1:12800 and 1:11143, respectively. In 100% of experimental group animals, the titers of antibodies to the vaccine antigen ranged from 1:6400 to 1:25600. Challenged primates and hamsters showed no focal infiltrative lungs changes specific to viral pneumonia, while placebo group demonstrated extensive lung damage and signs of viral pneumonia (pleurisy, cardiomegaly).

Accelerated viral elimination from URT was observed in vaccinated ferrets only. Infectious doses used for challenge of vaccinated primates and hamsters (10^6 and 10^2 infectious units, respectively) might have been high enough to allow systemic immunity induced by the injection of the peptide vaccine to stop URT mucosal cell infection.

In vaccinated animals, neutralizing antibodies were detected in low titers. However, neutralizing antibodies were found 2 weeks after vaccination in 100% of african green monkeys, but their titers decreased 4 weeks after vaccination. Virus-neutralizing titers (1:40 or more) were found in the majority of immunized ferrets (16 out of 18) and the most animals demonstrated seroconversion on day 14 after vaccination. Virus-neutralization was observed in 60% of hamsters

immunized with 260 μg of EpiVacCorona and in 50% of animals immunized with 85 μg of vaccine. However, vaccinated hamsters demonstrated 100% protection from pneumonia as well as monkeys. In ferret model the accelerated viral elimination from the URT was observed in vaccinated animals. Apparently, there are other factors of antiviral protection related to the presence of virus-specific antibodies, observed in all vaccinated animals at high titers. Non-neutralizing virus-specific antibodies also contribute to protection through opsonization of viral particles and infected cells and antibody-dependent cytotoxicity involving classical complement activation pathway and cell-mediated cytotoxicity.

The significance of cellular and antibody immune responses and their roles in preventing re-infections with coronavirus are actively studied. One of the assumptions is that a T-cell immune response is primarily required for the elimination of coronavirus. SARS-CoV-1-specific memory T-cells were shown to persist in the peripheral blood of patients up to 6 years after infection, even in the absence of memory B-cells [16, 17]. SARS-CoV-2 N protein, used as a carrier protein in EpiVacCorona vaccine, was shown to contain vast numbers of B-cell and T-cell epitopes [18-21] and recently it was also proposed as a valuable antigen for SARS-CoV-2 vaccines by several research groups [22-23]. Thus, the N-protein used in EpiVacCorona could contribute to protection not only as a source of T-helper epitopes but also evoking antigen-specific CD8+ T-cell responses and this question needs further investigation. However, alum hydroxide used here as adjuvant and the immunization route are suboptimal for stimulating cellular immune responses.

It is very important to point out the fact that vaccinated primates demonstrated decreased but relatively high titers of antibodies (to 1:3200) specific to the vaccine antigen 4 months after the first immunization with EpiVacCorona vaccine, regardless if high (1:25600) or minimal (1:6400) levels of vaccine-specific antibodies were achieved in first weeks after vaccination, which indicates the ability of the peptide vaccine to induce specific antibodies for a sufficiently long period.

Conclusions

Our studies have shown the EpiVacCorona vaccine to have a pronounced specific activity and to provide protection against SARS-CoV-2 in three species of animals: hamsters, ferrets, and non-human primates (African green monkeys and rhesus macaques). To generate a full-fledged immune response, a double intramuscular vaccination scheme was used while aluminum hydroxide was used as an adjuvant in the EpiVacCorona vaccine.

Additional information

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Contribution of authors. All authors attest they meet the ICMJE criteria for authorship.

Conflicts of Interest. ABR, MPB, EDD, IRI, EAN, OVP, OGP, IMS, EVG, RAM, AYP and EAR report holding a patent RU2738081 on peptide immunogens and vaccine composition. EAR and ESS declare employment at EpiVac LLC. All other authors declare no competing interests.

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