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ORIGINAL RESEARCH

Changes in nasal, pharyngeal and salivary secretory IgA levels in patients with COVID-19 and the possibility of correction of their secretion using combined intranasal and oral administration of a pharmaceutical containing antigens of opportunistic microorganisms

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Abstract

Background: Although extensive research has been conducted on the role of local immunity in patients with SARS-CoV-2, little is known about the production and concentrations of secretory IgA (SIgA) in different mucosal compartments. This article aims to assess the secretion of SIgA in the nasal and pharyngeal compartments and saliva of patients with COVID-19 and to investigate the possibility and efficiency of correction of their secretion using combined intranasal and oral administration of a pharmaceutical containing antigens of opportunistic microorganisms.

Methods: This study included 78 inpatients, aged between 18 and 60 years, who had confirmed COVID-19 with moderate lung involvement. The control group (n=45) received basic therapy, and the treatment group (n=33) was additionally administered the bacteria-based pharmaceutical Immunovac VP4 from day 1 to day 10 of hospitalization. SIgA levels were measured by ELISA at baseline and on days 14 and 30.

Results: No systemic or local reactions associated with Immunovac VP4 were reported. We observed a statistically significant reduction in the duration of fever and hospitalization in patients who received Immunovac VP4 compared with those from the control group (p=0.03 and p=0.05, respectively). Changes over time in SIgA levels in nasal swabs were found to be significantly different in the two treatment groups (F=7.9, p[78.0]<0.001). On day 14 of observation, patients in the control group showed a statistically significant reduction in SIgA levels from baseline (p=0.02), whereas patients in the Immunovac VP4 group had stable SIgA levels (p=0.07). On day 30 after the start of treatment, there was a statistically significant increase in SIgA levels in the Immunovac VP4 group compared with baseline (from 77.7 (40.5–98.7) µg/L to 113.4 (39.8–156.7) µg/L; p=0.05) and the levels measured on day 14 (from 60.2 (23.3–102.9) µg/L to 113.4 (39.8–156.7) µg/L; p=0.03). The control group showed a

statistically significant decrease in levels of nasal SIgA (to 37.3) on day 30 (p=0.007 for comparison with baseline values and p=0.04 for comparison with levels measured on day 14). Changes over time in SIgA levels measured in pharyngeal swabs were also different between the two treatment groups, and this difference reached statistical significance (F=6.5, p[73.0]=0.003). In the control group, this parameter did not change throughout the study (p=0.17 for a comparison between the levels measured on day 14 and the baseline values, and p=0.12 for a comparison between the levels measured on day 30 and the baseline values). In the Immunovac VP4 group, there was a statistically significant increase from baseline in SIgA levels on study day 30: from 1.5 (0.2–16.5) µg/L to 29.8 (3.6–106.8) µg/L (p=0.02). Changes over time in salivary SIgA did not show a significant difference between study groups (F=0.3, p[66.3]=0.75).

Conclusion: As part of combination therapy, the bacteria-based immunostimulant agent Immunovac VP4 increases SIgA levels in the nasal and pharyngeal compartments and induces clinical improvement. Induced mucosal immunity is central to the prevention of respiratory infections, particularly in patients with post-COVID-19 syndrome.

Keywords: bacteria-based pharmaceutical, COVID-19, immune therapy in COVID-19, mucosal immunity, SIgA.

Citation

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Introduction

Over the duration of the pandemic, most studies involving nasopharyngeal swab and saliva sampling were aimed at large-scale testing for SARS-CoV-2 and for virus characterization.[1] However, such specimens can also be used for the evaluation of mucosal immunity in patients with COVID-19. Whilst the volume of data on systemic immunity to SARS-CoV-2 continues to grow rapidly, there remains considerable uncertainty regarding the role of mucosal immunity in the protection against SARS-CoV-2.[2–5]

SARS-CoV-2 enters and starts replicating in cells of the upper airways, where ACE2 receptors are expressed at very high levels.[6,7] Prior research has shown that an early effective antibody response can change the clinical course of this infection as seen in influenza and chikungunya.[8,9] Some studies[10] showed that levels of most nasal antibodies against SARS-CoV-2, especially anti-RBD IgA, correlated with regression of systemic symptoms, but they did not observe any relationship between levels of these antibodies and the absence of respiratory symptoms. Although further investigation is required to determine the exact relationship between levels of nasal antibodies and clinical symptoms, higher baseline levels of anti-spike/RBD nasal antibodies were found to be correlated with a lower viral load; this trend was especially evident for anti-spike/RBD IgM. Early control of viral infection in the upper airways may inhibit dissemination of the virus and its replication in the lower respiratory tract and peripheral tissues, which accounts for milder systemic symptoms. These results are consistent with those reported by another study that also showed an important role of salivary anti-RBD IgA neutralizing antibodies in reducing the severity of clinical disease.[11]

It can be assumed that nasal antibodies play a key role in the onset of SARS-CoV-2 infection. The nasal antibody profile is different from the serum profile, especially in terms of secretory IgA and IgM (SIgA and SIgM, respectively). SIgA is mostly dimeric, whilst serum IgA is mainly monomeric. This might influence both viral neutralization and inflammatory response,[12–14] with the latter requiring further investigation.

Although extensive research has been conducted on the role of local and systemic immunity in patients with SARS-CoV-2 infection, little is known about the production and concentrations of SIgA in different mucosal compartments and their correlation with the course of COVID-19 and disease outcomes.

Saliva has been increasingly proposed as a diagnostic specimen source for SARS-CoV-2 detection and has been less often used to assess the role of this infection in the development of upper airway inflammation. IgA1 percentages in nasal secretions and saliva reach 80–90% and 60%, respectively.[15] A strong relationship has been observed between serum and salivary concentrations of IgM and IgG antibodies, whilst the correlation between IgA levels in serum and saliva was much weaker. This is explained by the fact that IgM and IgG primarily enter saliva from the blood, whilst salivary IgA are mainly secreted in the salivary glands as SIgA.[16] These

antiviral SIgA found in the mucosa were shown to be more effective than the serum monomeric form, which demonstrates their more potent neutralizing properties.[17] Therefore, salivary levels of SIgA may change over time in patients with COVID-19, which requires further evidence.

Little is known about ways to stimulate mucosal immunity in new SARS-CoV-2 infection. Before the COVID-19 pandemic, topical and systemic immunoactive agents with different mechanisms of action were widely used for the treatment and prevention of other respiratory viral infections, which reduced the risk of complications and recurrence of the disease.[18–24] This suggests that corrective immune-boosting therapy may also influence the outcome of COVID-19.

Currently, many studies have shown that different compartments included in the mucosal immune system, depending on many conditions, react differently to external stimulation. We have previously investigated and described the effect of combined nasal and subcutaneous administration of Immunovac VP4 on the mucosal immune system.[25] Herein, we evaluate the effect of Immunovac VP4 on various compartments of mucosal immunity with a combined intranasal and oral route of administration because previous studies have shown that the modulating effect of Immunovac VP4 depends on the route of its administration, and their combinations acquire new immunogenic properties.[26,27] Simultaneously, we were interested in finding the most effective, simple, and convenient way to use the immunomodulator, especially in relation to the area of the nasal compartment, which is the entrance gate of the SARS-CoV-2 virus.

Considering all of the above, the purpose of the current study is to assess the secretion of SIgA in the nasal and pharyngeal compartments and saliva of patients with COVID-19 and to investigate the possibility and efficiency of correction of their secretion using combined intranasal and oral administration of a pharmaceutical containing antigens of opportunistic microorganisms.

Methods

Clinical study design

The main objectives of the study were to evaluate the concentrations of total SIgA in different compartments of the upper respiratory tract in COVID-19 patients and to assess the effects of a bacteria-based immunostimulant on the secretion of SIgA in this patient population over the period between admission to hospital and discharge (from day 1 to day 30).

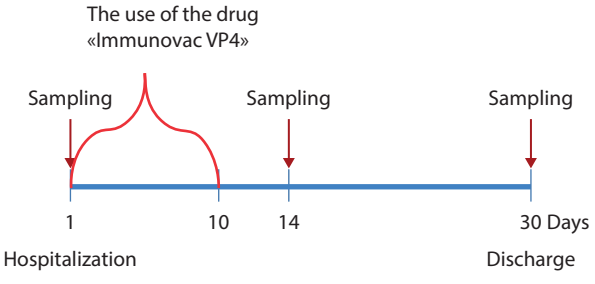
A total of 78 inpatients with COVID-19 were included in the study. They were divided into the following groups: group 1 (n=45) consisted of patients who received only background therapy in accordance with the clinical guidelines for the treatment of COVID-19 developed by the Ministry of Health of the Russian Federation, and group 2 (n=33) was made up of patients who received background therapy combined with Immunovac VP4 pharmaceutical, a bacteria-based immunostimulant. The general scheme of the study is shown in Figure 1.

This was a phase IV controlled randomized post-marketing study conducted in the Main Military Clinical Hospital of the National Guard Troops of the Russian Federation in 2021, a dedicated COVID-19 hospital in Moscow (Russian Federation). Patients were selected after medical tests, physical examination, and an assessment of the inclusion and exclusion criteria as well as the indications and contraindications for Immunovac VP4 as per the package leaflet. The selection of patients was also performed in accordance with the information provided in the formal Provisional Guidelines 'Prevention, diagnosis, and treatment of novel coronavirus infection (COVID-19)' developed by the Ministry of Healthcare of the Russian Federation. Patients were followed up for a minimum of 30 days. All treatment information, physical examination findings, and test results were reported using standard medical records (individual patient documentation).

Ethics approval and consent to participate

Treatment and study procedures were carried out in accordance with the Russian Provisional Guidelines 'Prevention, diagnosis, and treatment of novel coronavirus infection (COVID-19)' and clause 20 'Voluntary Informed Consent to Medical Intervention and Refusal of Medical Intervention' (Federal Law No. 323—, dated 1 November 2011 "On Fundamental Healthcare Principles in the Russian Federation" (as amended on 3 April 2017).

Figure 1. Scheme of the experiment and the use of Immunovac VP4 in experimental group during hospitalization.



The study protocol was approved on 26 November 2020 by the local Ethics Committee of the Federal State Budgetary Scientific Institution I.I. Mechnikov Research Institute of Vaccines and Sera (Russian Federation). The study was conducted in accordance with ethical principles and recommendations of the Declaration of Helsinki, the International Council for Harmonization's Good Clinical Practice guideline, Russian regulatory requirements and WHO. Written informed consent was obtained from the patients before enrolment in the study.

Patients

A total of 78 inpatients aged between 18 and 60 years who had confirmed COVID-19 infection with moderate lung involvement were included in the study. SARS-CoV-2 infection was confirmed by PCR of nasopharyngeal swabs. Upon recovery, a necessary condition for hospital discharge was the presence of a repeated negative PCR test for SARS-CoV-2. The patients with COVID-19 included in the study met the inclusion criteria. All of them received standard background therapy, which was selected according to the severity of their disease and as recommended by the Russian Provisional Guidelines 'Prevention, diagnosis, and treatment of novel coronavirus infection (COVID-19)'. It included favipiravir 200 mg (standard regimen), enoxaparin 0.4 mg/day, subcutaneously, dexamethasone 8–12 mg/day, and tocilizumab 400 mg/day (for patients with C-reactive protein <60 mg/L). Thus, 4 (8.9%) patients in the first group and 3 (9.1%) patients in the second group received tocilizumab.

Concomitant diseases in the entire cohort (*n*=78) were represented by simple chronic bronchitis in 17 (21.8%) patients, chronic gastritis in 15 (19.2%) patients and arterial hypertension in 12 (15.4%) patients. Diabetes mellitus and obesity occurred in 6 (7.7%) and 9 (11.5%) patients, respectively. There were no statistically significant differences in the prevalence of comorbidities between the study groups.

Group 1 (control group, *n*=45) consisted of both men and women (30/15); the mean age was 45.2±13.8 years. In group 1, 10 (22.2%) patients had chronic bronchitis, 9 (20%) patients had chronic gastritis, and 7 (15.6%) patients had hypertension. These patients received only background therapy. Group 2 (*n*=33, mean age 41.9±9.9 years) comprised men and women (24/9) who received Immunovac VP4 pharmaceutical, a bacteria-based immunostimulant, as an add-on to background therapy. This pharmaceutical was given starting on day 1 of hospitalization after careful consideration of all indications and contraindications as per the package insert. In group 2, 7 (21.2%) patients had chronic bronchitis, 6 (18.9%) patients had chronic gastritis and 5 (15.2%) patients had hypertension.

Some patients were withdrawn from the study because they refused to undergo certain tests (i.e. some data were missing at baseline, data were not corrected and not deleted). According to the intent-to-treat principle, analysis was performed on all patient data, and patients with missing data were not excluded from the analysis. Of note, data were analysed using a special method (linear mixed models), which effectively handles missing data.[28]

Samples were also taken from different compartments of the upper respiratory tract of healthy unvaccinated healthcare workers who had not been exposed to SARS-CoV-2 (*n*=10). The study parameters were measured in these samples; median values were calculated and considered as median reference values.

The clinical, laboratory and chest CT data of the study patients are shown in Table 1.

Inclusion criteria

Patients included had never been previously vaccinated against COVID-19, had confirmed SARS-CoV-2 infection (SARS-CoV-2 RNA detected in an upper respiratory tract swab by PCR and/or clinical and X-ray confirmation, indicative clinical signs and X-ray signs suggestive of viral lung damage; body temperature above 38°C at the onset; abnormalities on lung CT scan consistent with viral lung damage grade 2 CT scan and moderate lung involvement, 25–50%), and had provided signed and dated informed consent.

Exclusion criteria

Patients were excluded if they met any of the following criteria: concomitant diseases (lung abscess, pleural empyema, active tuberculosis); severe birth defects or serious chronic disorders, including exacerbations of chronic disorders, such as decompensated pulmonary, liver, renal, cardiovascular, neurological, mental or metabolic diseases, and stage IV or V chronic kidney disease; a history of cancer, including leukaemia, within the last 5 years; a history of positive HIV or hepatitis B or C test; use of immunoglobulin or blood transfusion within the last 3 months prior to the start of the study; long use (more than 14 days) of immunosuppressive or other immunomodulatory drugs within the last 6 months prior to the start of the study; any known or suspected immunosuppressive or immunodeficiency disorder or active autoimmune disease; any vaccination within the last month, including vaccination against SARS-Cov-2; existing pregnancy or lactation; simultaneous participation in another clinical study; and the patient's inability to comply with the study protocol requirements (as judged by the investigator).

Table 1. Clinical, laboratory and chest CT data of study participants.

Parameter	Total study population (*n*=78)	
	M (mean)±SD[1]	Me (median) (Q1–Q3)[2]
Age, years	44.7 ± 10.9	43 (37.2–49)
BMI, kg/m^2	29.5 ± 3.01	30 (27.1–32.3)
Duration of disease prior to admission, days	7.3 ± 1.64	7 (6–8)
Body temperature, ºC	37.5 ± 0.43	37.5 (37.1–37.8)
Respiratory rate, breaths/min	23.7 ± 1.25	24 (23–24)
SBP, mm Hg	124±9,5	121 (110–145)
DBP, mm Hg	83±8,6	80 (70–95)
Heart rate, beats/min	89.8 ± 7.94	88 (82–100)
SpO_2, %	92.8 ± 1.01	92.5 (92–93)
WBC, 10^9/L	6.5 ± 2.8	6.0 (4.3–7.5)
Platelets, 10^9/L	213.9 ± 78.39	189 (150–270)
Lymphocytes, 10^9/L	0.9 ± 0.37	0.8 (0.7–0.9)
Lymphocytes, %	15.3 ± 6.1	15.6 (11.9–21.8)
CRP, mg/L	66.7 ± 27.85	74 (42–96)
Fibrinogen, g/L	5.4 ± 1.37	5.4 (4.4–5.7)
D-dimers	0.6 ± 0.33	0.6 (0.4–0.9)
Chest CT, % lung involvement	44.7 ± 5.26	45 (39–50)

BMI, body mass index; CRP, C-reactive protein; CT, computed tomography; DBP, diastolic blood pressure; SBP, systolic blood pressure; SpO_2, blood oxygen saturation.

Immunovac VP4 pharmaceutical

Immunovac VP4 is a polyvalent vaccine based on the antigens of opportunistic microorganisms (a mixture of water-soluble antigens extracted from *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Escherichia coli*). This product is approved for subcutaneous use (Registration Certificate # ЛСР-001294/10 issued by the Ministry of Health of the Russian Federation on 24 February 2010) as well as nasal and oral use (Registration Certificate # ЛСР-001293/10 issued by the Ministry of Healthcare of the Russian Federation on 24 February 2010). It is manufactured by Scientific and Production Association for Immunological Preparations 'Microgen', a federal state unitary company (Ufa, Russian Federation).

Pharmacological properties

Immunovac VP4 is a bacteria-based immunostimulant, with a mechanism of action based on the activation of key effectors of innate and adaptive immunity. This pharmaceutical enhances phagocytic activity of macrophages; optimizes T cell counts and functional activity of lymphocyte subsets ($CD3^+$, CEM, $CD8^+$, $CD16^+$ and $CD72^+$); programmes $CD4^+$ T cells to proliferate and differentiate into T helper 1 cells; stimulates the production of IFNγ and IFNα; and improves the production of immunoglobulin isotypes by inhibiting IgE synthesis and inducing IgG, IgA and SIgA synthesis. It induces the production of antibodies to four opportunistic microorganisms whose antigens are included in the composition. It also provides cross protection against *Streptococcus pneumoniae*, *Haemophilus influenzae* and other pathogens due to the existence of common antigen components. In terms of clinical outcomes, vaccination reduces the rate of acute infections, duration of infection, severity of symptoms, risk of exacerbation of chronic diseases and amount of medication treatment.

This pharmaceutical is administered through a combined regimen of intranasal administration followed by oral administration. It can also be administered subcutaneously. Immediately before use, 2 mL of solvent (0.9% sodium chloride for injection or boiled water brought to 18–25ºC) is added to the vial with a syringe, and the

contents are mixed. The product is instilled into the nasal cavity using a medical dropper. For oral use, the required amount of pharmaceutical is drawn from a vial with a syringe and then transferred into a spoon.

Drug–drug interactions

The product can be used with other medications as a part of combination treatment. It can be administered along with antibiotics, antiviral, antifungal and antihistamine agents, bronchodilators, corticosteroids, and β-adrenoceptor agonists. Patients who receive immune therapy or immunoprophylaxis with Immunovac VP4 should not receive any other immunomodulatory agents within 1 month before this course of therapeutic or preventive treatment and within 3 months after its completion.

Schedule, dose and timing for vaccination

When prepared, the solution of Immunovac VP4 was administered to patients at a dose of 2 mL (20.0 mg) per os and 2 drops (1.0 mg) in each nostril daily from day 1 to day 10 of the hospital stay.

Data acquisition

For all patients, demographic data, body mass index, symptoms of the disease, physical examination findings, results of laboratory tests (complete blood count, C-reactive protein and blood coagulation profile), chest CT and concomitant diseases were assessed. Pulse oximetry was performed to detect respiratory failure and assess the degree of hypoxaemia.

The severity of respiratory failure was defined by the blood oxygen saturation level (SpO_2). Patients' nutritional status was assessed by body mass index. Pulse oximetry was performed using a pulse oximeter (series MD300C).

Chest CT was performed on a spiral CT scanner Aquilion TSX-101A (Toshiba Medical Systems, slice thickness 1 mm, pitch 1.5) on admission and after 10 days of treatment.

Sampling

In study groups 1 and 2, samples were taken from different compartments of the upper respiratory tract: nasal mucosal epithelial scrapings, pharyngeal epithelial scrapings and salivary gland secretions. Saliva was collected early in the morning before patients brushed their teeth and had a meal. Saliva was collected passively without any forceful coughing under the supervision of a physician.[29–31] Sampling was performed in two steps: on study day 1, before treatment was initiated; on study day 14, prior to discharge; and subsequently 30 days after the start of treatment.

Cytobrush sampling was performed in all patients to determine protein levels. Samples were collected using a type D brush (Yunona, Russian Federation) in three Eppendorf tubes with sodium chloride solution. The tubes were centrifuged at 2000 *g* for ~5 minutes to sediment epithelial cells and then refrigerated at +2–4°C until shipment to the laboratory, where the samples were examined within 24 hours after collection.

Methods

Levels of total SIgA in all biological fluids were measured using the enzyme-linked immunosorbent assay kit IgA secretory-IFA-BEST produced by Vector Best Company, Russian Federation (Cat. Number A-8668; Reg. number FSR 2010/07853). Plates were read using a Multiskan Ascent ELISA microplate photometer (Thermo Electron Corporation, Finland). The method of determining the concentration of immunoglobulins implemented in this set is the enzyme-linked immunosorbent assay based on a two-step sandwich enzyme immunoassay using monoclonal antibodies against the secretory component linked to the α-chain of IgA. All procedures for determining the concentrations of SIgA were carried out in strict accordance with the instructions attached to the kit. The main stages of the measurements reflected in these instructions were as follows: calibration standards with known concentrations of SIgA and the samples were added to wells of a plate coated with an anti-SIgA monoclonal antibody. The plate was then incubated according to the test kit instructions. The intensity of the developing colour is proportional to the concentration of SIgA in the sample and was determined on a spectrophotometer at a wavelength of 450 nm. The concentration of SIgA was calculated using the standard curve and the measured optical density values.

These tests were performed using certified equipment provided by the Research Equipment Sharing Center of the Federal State Budgetary Scientific Institution I.I. Mechnikov Research Institute of Vaccines and Sera. Table 2 shows the number of patients who underwent SIgA tests.

Statistics

The normality of the distribution of quantitative variables was tested using Shapiro–Wilk normality test. Most variables were found to have a non-normal distribution; therefore, descriptive statistics for quantitative variables included the median and interquartile range, Me(Q1–Q3). The 95% CIs were calculated for the differences between the medians at the two time points.

Changes over time in SIgA levels were compared between the study groups using a linear mixed-effects model, where group and time points were fixed factors, and patients were random factors. This model was created in the lme4 package.[32] When the model was created, goodness-of-fit tests (the normality of distribution and homogeneity of variance in residuals) were

Table 2. Number of patients who had secretory IgA tests.

Group	Number of patients (*N*)								
	Pharyngeal swabs			Nasal swabs			Saliva samples		
	Day 1	Day 14	Day 30	Day 1	Day 14	Day 30	Day 1	Day 14	Day 30
VP4	29	23	12	28	25	13	29	23	14
Control	35	33	34	34	37	32	25	33	29

conducted using the DHARMa package.[33] If these goodness-of-fit tests showed some problems, a Box–Cox transformation was applied to the initial dataset, then a corrected model was built and goodness-of-fit tests were run on the transformed data. If the discrepancy was still statistically significant, a linear mixed-effects model was built using the robust estimation method in the robustlmm package.[34] For parametric estimation in the linear mixed-effects model, pooled results for three time points are presented, which were obtained by applying type III ANOVA with Kenward–Roger approximation for degrees of freedom. For non-parametric estimation, the results were obtained for each time point separately using Satterthwaite's approximation for degrees of freedom. In both cases, tests were performed using the lmerTest package.[35] *Post hoc* tests were performed using corresponding contrasts in the calculated linear mixed-effects model with a Benjamini–Krieger–Yekutieli correction.[36]

Individual quantitative variables were compared between the study groups using the Mann–Whitney test. The one-sample Wilcoxon test was used to compare the medians of quantitative parameters to the estimated medians.

Statistically significant differences were defined as $p<0.05$. Calculations and graphics were carried out using GraphPad Prism (v.9.3.0, license GPS-1963924) and the statistical programming environment R (v.3.6, license GNU GPL2).

Results

No systemic or local reactions to Immunovac VP4 were reported during the clinical observation.

A Kaplan–Meier analysis showed a statistically significant reduction in the duration of hospitalization in the group of patients receiving Immunovac VP4 compared with the control group: 16 (11–20) days *versus* 19 (16–21) days; $p=0.05$.

Analysing the clinical parameters, we drew attention to the decrease in the duration of fever whilst taking Immunovac VP4. However, further studies on a larger sample of patients are needed to clarify the identified clinical effect. In the Immunovac VP4 group, there was a reduction in the duration of fever compared with the control group. In patients with moderate pneumonia who received only background therapy, fever persisted for 4 (95% CI 0.5–6) days, whilst in patients who received background therapy along with Immunovac VP4, it lasted for 2 (95% CI 0.5–3) days; these differences were statistically significant (Figure 2).

In patients with COVID-19, changes over time in SIgA levels measured in nasal swabs were found to be significantly different in patients with moderate pneumonia between the two treatment groups ($F=7.9$, $p[78.0]<0.001$) (Figure 3). On day 14 after the start of the observation, there was a statistically significant decrease in this parameter compared with the baseline in the control group (from 96.3 µg/L (58.6–167.5) to 59.0 µg/L (21.9–138.1); $p=0.02$), whilst in patients who received Immunovac VP4 in addition to background therapy, SIgA levels did not change during

Figure 2. Kaplan–Meier curves of the duration of fever in patients with moderate COVID-19-associated lung involvement.

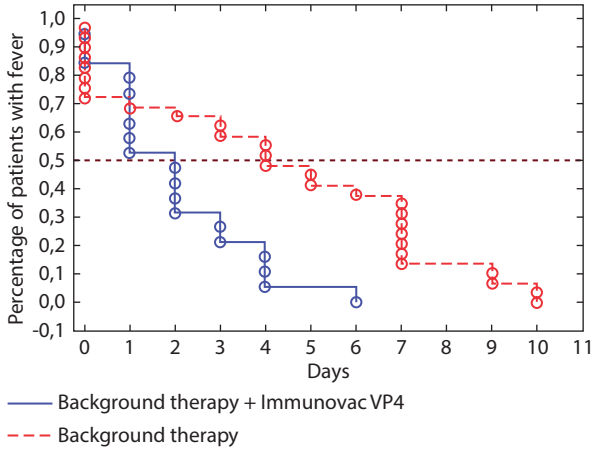
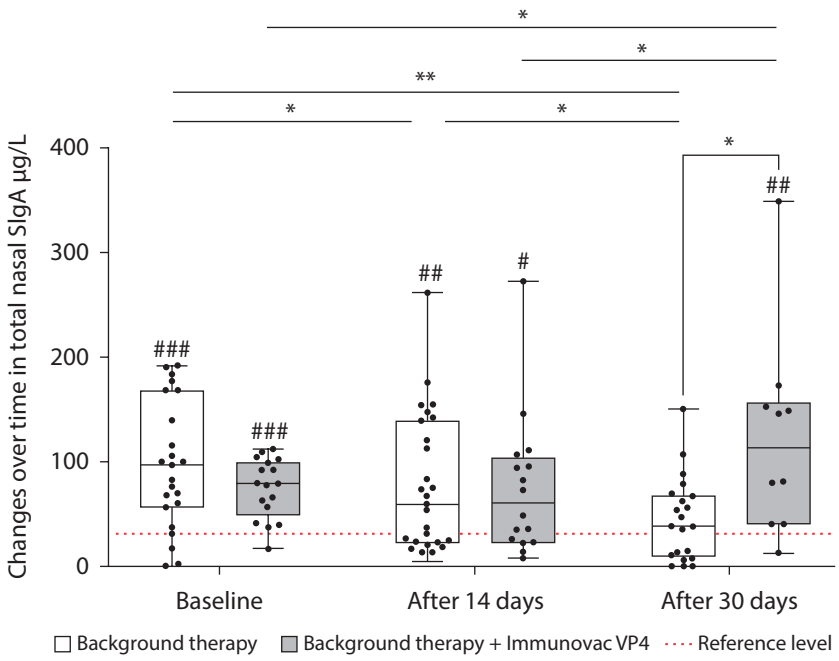


Figure 3. Changes over time in nasal SIgA in patients with COVID-19 with moderate lung involvement who received background therapy alone or in combination with Immunovac VP4 (at baseline and on days 14 and 30); individual values, median, interquartile range, minimum and maximum values. *$p \leq 0.05$, **$p < 0.01$, a linear mixed-effects model was used, and a Benjamini–Krieger–Yekutieli correction was applied for multiple comparisons. ##$p < 0.01$, ###$p < 0.001$ compared with the reference values (in healthy unvaccinated study participants), the one-sample Wilcoxon test was used.

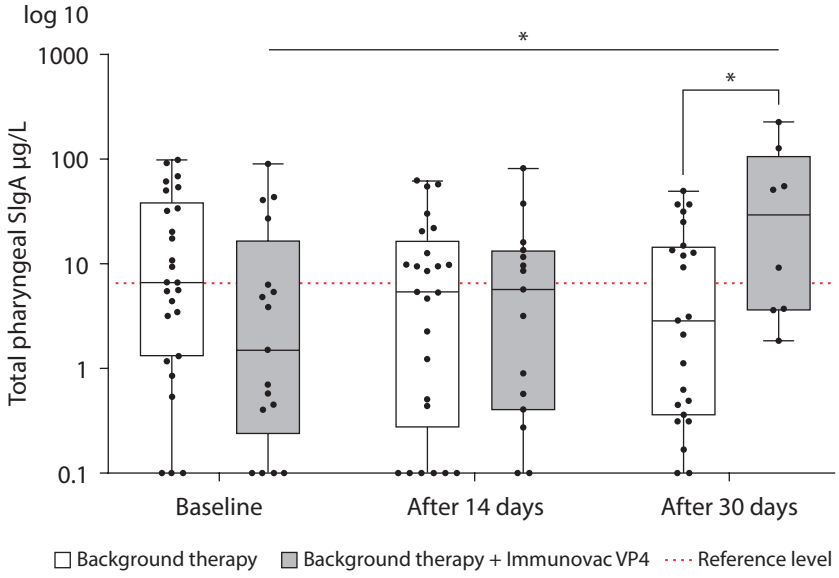


this period (p=0.07). On day 30 after the start of treatment, there was a statistically significant increase in SIgA levels compared with baseline in the Immunovac VP4 group (from 77.7 µg/L (40.5–98.7) to 113.4 µg/L (39.8–156.7); p=0.05) and to levels measured on day 14 (from 60.2 µg/L (23.3–102.9) to 113.4 µg/L (39.8–156.7); p=0.03). In contrast, patients who received only background therapy showed a statistically significant decrease in nasal SIgA on day 30 of observation (37.3 µg/L (8.4–66.9)) compared with baseline (99.9 µg/L (58.6–178.0); p=0.007) and day 14 of treatment (59.0 µg/L (21.9–138.1); p=0.04). The difference between the median levels of SIgA in nasal scraping on day 30 and baseline (delta) in the VP4 group was +22.1 µg/L (95% CI 1.8–89.8) *versus* −60 µg/L (95% CI −115.4 to −3.5) in the control group, and the difference in the delta between the groups was statistically significant (p=0.003). Thus, on day 30 after the start of treatment, patients receiving Immunovac VP4 along with background therapy had statistically significantly higher levels of SIgA than patients who received only background therapy (113.4 µg/L (39.8–156.7) *versus* 37.3 µg/L (8.4–66.9); p=0.05). In the Immunovac VP4 group, the levels of nasal SIgA were higher than the reference values throughout the study period (p<0.001 for comparison against baseline, p=0.03 for comparison against the values measured on day 14, and p=0.01 for comparison against the values measured on day 30).

Changes over time in SIgA levels measured in pharyngeal swabs were different between the two treatment groups (F=6.5, p[73.0]<0.001), and this difference reached statistical significance (Figure 4). In the group of patients receiving only background therapy, this parameter did not significantly change throughout the study (p=0.17 for comparison between the levels measured on day 14 and the baseline values and p=0.12 for comparison between the levels measured on day 30 and the baseline values). In contrast, patients receiving Immunovac VP4 along with background therapy showed a statistically significant increase from baseline in pharyngeal SIgA on study day 30 (from 1.5 µg/L (0.2–16.5) to 29.8 µg/L (3.6–106.8); p=0.02).

On day 30, the delta (change from baseline) of this parameter was +27.8 µg/L (from +0.3 to +221.7) in the

Figure 4. Changes over time in pharyngeal SIgA in patients with COVID-19 with moderate lung involvement who received background therapy alone or in combination with Immunovac VP4 (at baseline and on days 14 and 30); individual values, median, interquartile range, minimum, and maximum values. *$p \leq 0.05$, a linear mixed-effects model was used, and a Benjamini–Krieger–Yekutieli correction was applied for multiple comparisons.



Immunovac VP4 group *versus* −0.5 µg/L (from −14.2 to +2.8) in the control group (p=0.003). Therefore, on day 30 after the start of treatment, patients receiving Immunovac VP4 had higher levels of SIgA in pharyngeal swabs compared with patients in the control group, and this difference was statistically significant (29.8 µg/L (3.6–106.8) *versus* 2.9 µg/L (0.4–14.8); p=0.05). In either of the study groups, these levels did not statistically significantly deviate from the reference value.

Throughout the study period, the groups did not show a statistically significant difference in terms of levels of salivary SIgA (Figure 5; F=0.2, p[41.6]=0.65) or their changes over time (F=0.3, p[66.3]=0.75).

Throughout the study period, this parameter was statistically significantly higher than the reference value.

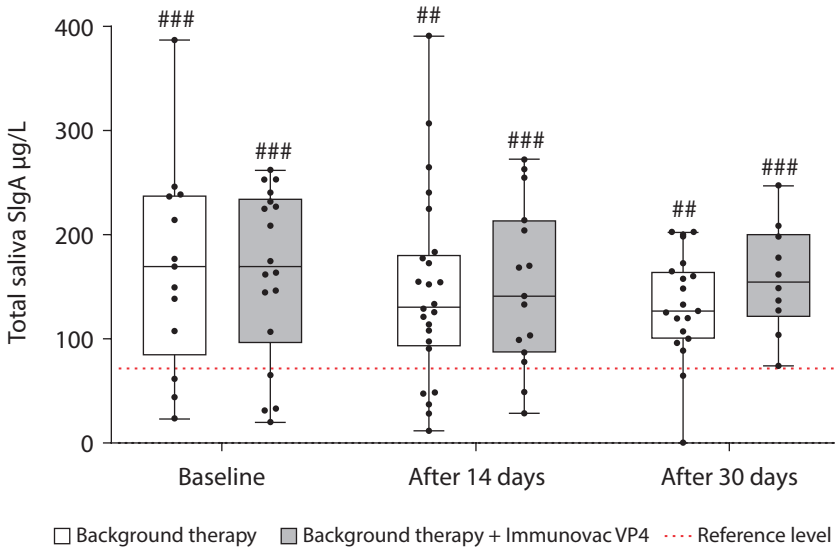
Discussion

Prior studies have shown that vaccines delivered by applying antigens to the mucosal surface eliminate the risk of transmitting not only SARS-CoV-2 but also other infections.[37] The clinical and pharmacological properties of Immunovac VP4 allowed us to suggest that this nasal preparation would promote the production of SIgA primarily in the upper and lower respiratory tract, where SARS-CoV-2 can be neutralized and eliminated without causing inflammation. It can be assumed that the detection of IgA in upper airway secretions can also be the most informative way of assessing the immune response to SARS-CoV-2 induced by either natural infection or this nasal preparation.[38,39] We demonstrated the restoration of total SIgA levels in patients who had been hospitalized with confirmed COVID-19, had moderate lung involvement and had received appropriate background therapy on day 30 after admission to hospital (on days 15–16 after discharge). By this time point, their SIgA levels returned to those seen in healthy unvaccinated individuals with no history of COVID-19. Undoubtedly, the normalization of SIgA is extremely important for preventing other diseases, and measuring their levels 2 weeks after discharge is critical for assessing the risk of other infections.

Throughout the study period, levels of salivary SIgA were higher in patients with COVID-19 than in healthy individuals who had not been exposed to SARS-CoV-2. Levels of SIgA in pharyngeal swabs did not significantly change in patients who received only background therapy and, in this group, they did not differ from the levels observed in healthy individuals.

If we remember the pathogenic mechanisms that underlie COVID-19-induced inflammation, it becomes clear why we observed high levels of SIgA in salivary gland

Figure 5. Changes over time in salivary SIgA in patients with COVID-19 with moderate lung involvement who received background therapy alone or in combination with Immunovac VP4 (at baseline and on days 14 and 30); individual values, median values and their 95% confidence intervals. ##p<0.01, ###p<0.001 for comparison against the reference values (in healthy unvaccinated study individuals), the one-sample Wilcoxon test was used.



secretions and nasal swabs of patients with COVID-19 on admission.[40–42] The main role of salivary immunoglobulins is long known: they inactivate various pathogens, such as bacteria, fungi and viruses as well as some microbial toxins, by binding and/or agglutinating these particles.[43] Such binding may prevent the adhesion of microorganisms and their toxins to the mucosal epithelium and result in their digestion in the stomach.[44,45] The persistence of high levels of salivary SIgA in patients with COVID-19, including those who received a combination of background therapy and the immunostimulant Immunovac VP4, even at 2 weeks after hospital discharge is an indirect sign of a persistent systemic inflammatory response typical for post-COVID-19 syndrome.

Saliva can be called a multicomponent biological fluid. In the mouth, ductal saliva of several salivary glands is blended and supplemented with many constituents that originate from intact or destroyed mucosal cells, immune cells and oral microorganisms. Blood constituents also enter the oral cavity via gingival crevicular fluid and intraoral bleeding. Consequently, a complex mixture of a high variety of molecules results in the oral cavity, frequently called mixed saliva.[46] The protein composition of saliva and the production of specific and non-specific antibodies may also be under the influence of ACE2 expression in different compartments of the oral mucosa, epithelial cells of the salivary glands, and the lung alveolar epithelium,[47,48] which results in long-term persistence of high levels of SIgA in saliva samples of patients with a history of COVID-19.

Comparison of the changes over time in SIgA levels in pharyngeal and nasal swabs of patients with COVID-19 who received background therapy alone or combined with Immunovac VP4 showed that the administration of immunostimulant increased SIgA levels in pharyngeal swabs 1 month after the start of treatment. Similar changes were observed for SIgA levels in nasal swabs: 30 days after the start of treatment, they were comparable to those seen in the healthy individuals (control group) (p=0.16) and remained higher in patients who received Immunovac VP4 along with background therapy (p=0.003).

It can be assumed that activated production of SIgA in the pharyngeal and especially nasal compartments in patients with a history of COVID-19 can influence the risk of superinfection, reinfection and bacterial complications of respiratory infections later because, amongst all immunoglobulin isotypes induced by nasal and oral Immunovac VP4, SIgA is central to preventing mucosal involvement in the infectious process. The correlation between SIgA levels and the level of protection against influenza A virus confirms this assumption.[49] IgA appears to mediate this protection by neutralizing free viral particles and/or their destruction inside cells

and through the activation of antiviral mechanisms in infected cells.[50,51] However, further investigations are required to determine the long-term clinical effects and role of SIgA induced by Immunovac VP4.

The exact mechanism of the action of Immunovac VP4 in various diseases is not yet completely understood. In a previous study,[52] an increase in IL-5 secretion was observed under the influence of Immunovac VP4, leading to enhancement of the synthesis of immunoglobulins. Similarly, an increase in IL-6 actively enhanced the synthesis of IgA in nasal lymphoid tissue (NALT) cells. Simultaneously, increased production of IL-12 enhanced the cellular immune component of the effect of Immunovac VP4, stimulating the maturation of $CD8^+$ cells and enhancing the action of natural killer cells. An increase in the number of T helper cells and an intensification of the processes of activation of B lymphocytes and dendritic cells, which can occur judging by the increase in the number of CD4, CD19(B4) and CD20(B1) receptors in NALT under the influence of Immunovac VP4, can also contribute to an increase in the synthesis of antibodies.[53–55]

As Immunovac VP4 increases the levels of TLR2, TLR4 and TLR9, we can assume that the observed clinical effects may also be due to the activation of the innate immune system. In addition, through TLR4 and TLR9, it is also possible to stimulate the maturation of immunological regulatory B memory cells.[53,56] Given that the number of T lymphocyte markers in NALT lymph nodes under the influence of Immunovac VP4 increases by 32 times, it can be assumed that the pool of these lymphocytes combines various clinical effects (including a potential antiviral effect) of Immunovac VP4 into a single complex.[57]

Importantly, adding an immunostimulant to background therapy for patients with COVID-19 was accompanied by many positive effects, including a reduced duration of fever, which influenced the duration of the recovery period. We certainly cannot associate these changes in the clinical picture with the reported difference in SIgA levels without confirming the difference in the level of secreted anti-SARS-CoV-2 SIgA antibodies; this requires further investigation. However, there is still the possibility that Immunovac VP4 has a beneficial effect on other parameters of innate and adaptive immunity, improving the clinical picture of the disease.[53,58]

It should also be noted that, as a result of comparing the data obtained in this study with the results obtained previously,[25] we concluded that the combined intranasal and subcutaneous administration of Immunovac VP4 does not have pronounced advantages compared with combined intranasal and oral administration in terms of their immunogenicity; however, we prefer the latter due to its simplicity and safety.

Conclusion

This study demonstrated the positive effects of the use of Immunovac VP4, containing antigenic components of *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Escherichia coli*, which has immune-inducing properties. These effects included the restoration of SIgA levels in the nasal and pharyngeal compartments in patients with COVID-19 similar to that previously observed in patients with other infections treated with this pharmaceutical.[59,60]

Theoretically, the mechanism of action of this product is due to the activation of key effectors of innate and adaptive immunity mediated by stimulation of the Toll-like receptor network. Stimulation of innate immunity with microbe-derived products containing conservative microorganism-specific pathogen-associated molecular patterns, which are recognized by immune receptors, results in rapid restoration of one of the important parameters (SIgA) of mucosal immunity in the post-COVID-19 period and the development of protection against different pathogens. These effects lead to pathogen recognition (signal of threat), activation of effector defence mechanisms within several hours, and initiation of the processes that cause the elimination of the pathogen and induce the formation of protective (adaptive) immunity within 7–14 days.[61]

As part of a combination therapy, the bacteria-based immunostimulant agent Immunovac VP4 increases the levels of SIgA in the nasal and pharyngeal compartments and induces clinical improvement. Induced mucosal immunity is central to the prevention of respiratory infections, particularly in patients with post-COVID-19 syndrome.

Contributions: MK – Conceptualization and methodology; OS – Conceptualization; AC – Conceptualization; NA – Investigation; VO – Resources; EK – Investigation; DP – Project administration; VT – Resources; AV – Formal analysis; VG – Writing original draft; KM – Writing, reviewing and editing; NK – Writing, reviewing and editing; IB – Writing, reviewing and editing; AK – Visualization and validation. All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this article, take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

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