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Humoral and Cellular Immune Response to SARS-CoV-2 S and N Proteins

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Abstract—The pandemic of a new coronavirus infection that has lasted for more than 3 years, is still accompanied by frequent mutations in the S protein of SARS-CoV-2 and emergence of new virus variants causing new disease outbreak. Of all coronaviral proteins, the S and N proteins are the most immunogenic. The aim of this study was to compare the features of the humoral and T-cell immune responses to the SARS-CoV-2 S and N proteins in people with different histories of interaction with this virus. The study included 27 individuals who had COVID-19 once, 23 people who were vaccinated twice with the Sputnik V vaccine and did not have COVID-19, 22 people who had COVID-19 and were vaccinated twice with Sputnik V 6-12 months after the disease, and 25 people who had COVID-19 twice. The level of antibodies was determined by the enzyme immunoassay, and the cellular immunity was assessed by the expression of CD107a on $CD8^{high}$ lymphocytes after recognition of SARS-CoV-2 antigens. It was shown that the humoral immune response to the N protein was formed mainly by short-lived plasma cells synthesizing IgG antibodies of all four subclasses with a gradual switch from IgG3 to IgG1. The response to the S protein was formed by short-lived plasma cells at the beginning of the response (IgG1 and IgG3 subclasses) and then by long-lived plasma cells (IgG1 subclass). The dynamics of antibody level synthesized by the short-lived plasma cells was described by the Fisher equation, while changes in the level of antibodies synthesized by the long-lived plasma cells were described by the Erlang equation. The level of antibodies in the groups with the hybrid immunity exceeded that in the group with the post-vaccination immunity; the highest antibody content was observed in the group with the breakthrough immunity. The cellular immunity to the S and N proteins differed depending on the mode of immune response induction (vaccination or disease). Importantly, the response of heterologous $CD8^+$ T cell to the N proteins of other coronaviruses may be involved in the immune defense against SARS-CoV-2.

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Keywords: COVID-19, SARS-CoV-2, N protein, S protein, antibodies, vaccination, hybrid immunity, cellular immunity, breakthrough immunity

INTRODUCTION

Recent pandemic of the new coronavirus infection that has lasted for more than 3 years, is still accompanied by frequent mutations in the S protein of SARS-CoV-2 (acute respiratory syndrome coronavirus 2) and emergence of new virus variants causing further disease outbreaks, suggesting that this infection will remain with humanity for many more years. Although it might become less severe, the fight against it may turn into a permanent problem [1]. The most immunogenic of coronavirus proteins are the S and N proteins [2] that induce generation of large amounts of antibodies in response to the SARS-CoV-2 infection [3, 4]. Anti-SARS-CoV-2 vaccines target the S protein, since antibodies against this protein provide strong protection against the infection. However, frequent mutations in

Abbreviations: BAU, binding antibody unit; COVID-19, coronavirus infectious disease 19; SARS-CoV-2, acute respiratory syndrome coronavirus 2.

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the S protein have led to a decrease in the efficacy of existing vaccines [5, 6]. The interest of researcher in the S protein has left the N protein somewhat in the shadows. This protein is highly conserved among coronaviruses and is one of the most abundant structural proteins in the virus-infected cells [7]. The main function of the N protein is to package the viral genomic RNA into a long helical ribonucleocapsid complex and participate in the virion assembly through interaction with the viral genome and membrane protein M [8]. The location of the N protein in the center of the coronavirus virion explains why even high levels of antibodies against this protein do not protect against the disease, as the antibodies cannot enter the assembled virion and contact the N protein. Moreover, the effects of anti-N protein antibodies are poorly understood [9]. At the same time, the N-protein is a representative antigen in the T-cell response against the infection. It was shown that the T-cell response formed against SARS-CoV-1 persists for many years [10-12]. Since the N protein is highly conserved, it contains epitopes that could generate T-cell immune responses that are cross-reactive across SARS-CoV-2 and other human coronaviruses [13].

The purpose of this study was to compare the characteristics of humoral and T-cell immune responses to the SARS-CoV-2 S and N proteins in people with different histories of interaction with the virus.

MATERIALS AND METHODS

Analyzed cohorts and collection of biological material. Simple open-label comparative study included 97 adult volunteers aged 18-73 years. Of these, 27 people had a history of mild to moderate COVID-19 (coronavirus disease 19) confirmed by at least one positive PCR test (group 1; post-infectious immunity). They were examined 4 to 7 times over 1-18 months from the disease onset. Group 2 (post-vaccination immunity) consisted of 23 people who were vaccinated twice with the Sputnik V vaccine and did not have COVID-19. Group 3 (hybrid immunity) included 22 people who had COVID-19 and were vaccinated twice with Sputnik V 6-12 months after the disease. Group 4 (breakthrough immunity) included 25 people who had COVID-19 twice: the first time in 2020-2021 and again in 2022 (omicron strain). Blood for the study was taken from the ulnar vein into two vacuum tubes (4 ml each) containing either heparin (cellular immunity studies) or coagulation activator and gel for isolating blood serum (assessment of the humoral immune response to the SARS-CoV-2 antigens), respectively. The study was approved by the Ethics Committee of the Gabrichevsky Research Institute for Epidemiology and Microbiology (protocol no. 58; December 15, 2021). Informed voluntary consent was obtained from each participant included in the study.

Evaluation of antibody levels. Blood serum was obtained by centrifugation, transferred into Eppendorf tubes, and stored at –70°C until the study. The antibody content was determined by the enzyme immunoassay using the SARS-CoV-2-IgG quantitative-ELISA-BEST kit (JSC Vector-Best, Novosibirsk, Russia) for the anti-S protein antibodies and N-CoV-2-IgG PS kit for the anti-N protein antibodies (Saint-Petersburg Pasteur Institute, St. Petersburg, Russia). The subclasses of IgG antibodies to the SARS-CoV-2 antigens were studied using a previously developed modification of ELISA method [14, 15]. Briefly, we used 96-well panels with adsorbed full-length S antigen from the SARS-CoV-2-IgG quantitative-ELISA-BEST kit or with the N protein from the N-CoV-2-IgG PS kit. Instead of anti-IgG conjugates included in the kit, peroxidase-labeled anti-IgG1, IgG2, IgG3, and IgG4 monoclonal antibodies (Polignost, St. Petersburg, Russia) were used at a concentration of 1 μg/ml. All other stages of the assay were carried out according to the kit instructions.

Assessment of cellular immunity. Mononuclear cells were isolated from heparinized blood under sterile conditions using gradient centrifugation (ρ = 1.077; PanEco, Russia) and washed from platelets. The cells were transferred to the wells of a sterile 96-well plate (2.5×10^5 cells per well) containing RPMI-1640 medium supplemented with 2 mM L-glutamine, gentamicin, and 10% fetal calf serum (PanEco). Monensin (final concentration, 10 μM) and PE-Cy5-labeled monoclonal antibody against CD107a (final dilution, 1 : 100) (control samples) were added to the wells; the final volume in the well was 200 μl. In experimental samples the cells were stimulated with the SARS-CoV-2 S and N antigens using the plates from the corresponding antibody ELISA kits (see above) that had the S or N proteins adsorbed at the bottom of the wells. Since the ELISA plates were not sterile, they were sterilized by ultraviolet irradiation for 30 min before the experiment. All ingredients were added equally to both the experimental and control wells, according to the previously developed method. Experimental and control samples were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 20 h, transferred into the tubes for cytofluorimetry, washed with CellWash (300*g* for 5 min), stained with FITC-labeled antibodies against CD8 for 20 min in the dark at 4°C, washed again under the same conditions, and immunophenotyped using a BD FACS CantoII flow cytometer (Becton Dickinson Technologies and Software, USA). When analyzing the results, we established the lymphoid gate and the gate for lymphocytes highly expressing the CD8 antigen ($CD8^{high}$) within it and calculated the percentage of $CD8^{high}CD107a^{+}$ cells, i.e., cytotoxic T lymphocytes that recognized the S or N antigens and responded by

releasing the content of cytotoxic granules (cytotoxic attack). The level of 1% was considered as the limit of spontaneous expression of the CD107a molecule on $CD8^{high}$ lymphocytes [15].

Statistical analysis. The normality of data distribution was determined by the Kolmogorov–Smirnov method. The levels of anti-S protein and anti-N protein IgG antibodies did not show the normal distribution. The antibody content was expressed in antibody binding unit (BAU) per ml and presented as the median (1st-3rd quartile) [Me (LQ-HQ)]. The differences between the groups were assessed using the Mann–Whitney U test. The percentage content of IgG subclasses and cellular immunity parameters showed normal distribution and were presented as mean ± standard error of mean (M ± SEM). The correlations were assessed using the Pearson method. The differences were considered significant at $p < 0.05$.

Modeling. The data on the changes in the content of anti-S protein and anti-N protein IgG antibodies over time elapsed from the disease onset were approximated. The observed changes corresponded to a distribution with the following characteristics: the initial value corresponded to the origin of coordinates; a sharp increase in the function value to a certain maximum followed by its smooth decrease. As is known from mathematical statistics, this type of distribution is described by the Fisher and Erlang distributions. These functional dependencies are widely known in statistical analysis, probability theory, and biology and belong to the Pearson type III distribution group (gamma distributions). Our study examined two of them. The first one was the Erlang distribution (1)

$$f(x;\, k,\, \lambda,\, c) = c \cdot \frac{\lambda^k \cdot x^{k-1} \cdot e^{-\lambda x}}{(k-1)!}, \qquad (1)$$

where k is the shape parameter and λ is the rate parameter. The normalization coefficient c was introduced to scale the function values. The combination of k and λ determines the graph extremum position, the function value at the extremum point, and the function inflection smoothness.

The second one was the Fisher distribution (2):

$$f(x;\, n,\, m,\, c) = \begin{cases} c \cdot \dfrac{x^{\frac{n}{2}-1}}{\left(1 + \frac{n \cdot x}{m}\right)^{\frac{n+m}{2}}} & x \geq 0 \\ 0 & x < 0 \end{cases}, \qquad (2)$$

where coefficients n and m affect the shape of the graph and the position and value of the function maximum; the coefficient c is also normalizing.

The Erlang distribution is characterized by a smoother increase and decrease in the function values, while the Fisher distribution allows to describe the functions whose values drop sharply after reaching the maximum and then smoothly and asymptotically tend to zero.

In this study, the general form of the approximating function was chosen based on the nature of observed dependencies. We used an algorithmic process to sort through the coefficient values to achieve the best estimate of the standard deviation of the resulting approximation curve from the experimental data.

RESULTS

The changes in the content of IgG antibodies against SARS-CoV-2 antigens in the blood serum of patients who had recovered from COVID-19 (group 1) are presented in Fig. 1. The figure shows that the IgG antibodies recognizing the N protein (curve *1*) appeared earlier and their concentration increased faster in the blood with a sharp peak at 1033.2 (807.04-1215.6) BAU/ml 3 months after the disease onset, flowed by a rapid decrease to 75.5 (25.5-182.2) BAU/ml after 18 months (kit cut-off value, 33.5 BAU/ml). The content of anti-S protein antibodies (curve *2*) increased more slowly and reached a plateau at 819.5 (614.7-1538.3) BAU/ml 4 months after the disease onset, where it remained for a year, and then decreased to 551.6 (372.5-757.5) BAU/ml by 18 months. The curve reflecting the concentration of anti-N antibodies was well approximated by the Fisher distribution (Fig. 1, curve *3*) according to the formula (3):

$$f_{N\text{-}protein}(x) = 8 \cdot 10^8 \cdot \frac{(0.2x)^{5.5}}{(1 + 0.52x)^9}. \qquad (3)$$

Table 1 compares the data obtained by analyzing the content of anti-N protein IgG antibodies in the blood serum of individuals who had recovered from COVID-19 and the results calculated using formula (3). The experimental and calculated data differ by less than 15%, while the calculation results strictly fall into the LQ-HQ interval for all studied time points.

The attempts to approximate curve *2* reflecting the concentration of anti-S protein antibodies with any known distribution were unsuccessful. We assumed that the curve is the result of two processes: formation of early antibody response to the S protein by short-lived plasma cells and generation of antibodies by long-lived plasma cells (curves *4* and *5* in Fig. 1, respectively). Both formation and death of plasma cells are reflected in the concentration of antibodies they produce. This process has the following properties: the initial value corresponds to the origin of coordinates; the antibody concentration then sharply increases to a certain maximum value followed by a smooth decrease in the function value. As is known from mathematical statistics, this curve can be described by the Fisher and Erlang distributions. Therefore, it was necessary to adjust the two distributions so that their sum corresponded to curve *2* in Fig. 1. In this case, formation

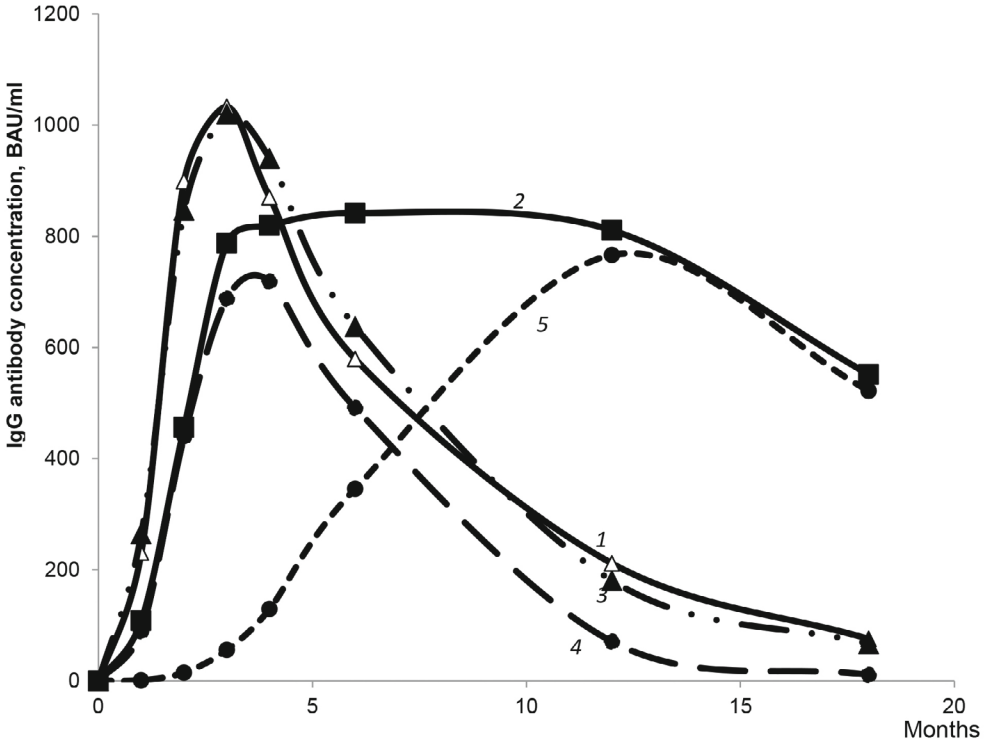

Fig. 1. The content of IgG antibodies to SARS-CoV-2 virus antigens. Curves: *1*) IgG antibodies to the N protein (experimental data); *2*) IgG antibodies to the S protein (experimental data); *3*) Fisher approximation of the content of IgG antibodies against the N protein (short-lived plasma cells); *4*) Fisher approximation of the content of IgG antibodies against the S protein (short-lived plasma cells); *5*) Erlang approximation of the content of IgG antibodies against the S protein (long-lived plasma cells).

Table 1. Comparison of the experimental data on the content (BAU/ml) of IgG antibodies against SARS-CoV-2 N protein in the blood serum from recovered individuals and the results of calculations using formula (3)

Time from the disease onset	IgG antibodies against N protein [Me (LQ-HQ)]	Calculation according to the Fisher formula (3)	Deviation of the calculated value from the experimental value, %
1 month	230.9 (118.4-430.6)	264.34	–14.5%
2 months	899.2 (497.8-1225.9)	846.74	5.83%
3 months	1033.2 (807.04-1215.6)	1020.38	1.2%
4 months	870.1 (319.7-3790.36)	940.00	–8.0%
6 months	579.3 (374.3-2524.9)	637.55	–10.1%
12 months	211.5 (116.9-507.3)	180.55	14.6%
18 months	75.5 (25.5-182.2)	66.76	11.6%

of antibodies by the early producers was also approximated with the Fisher distribution (curve *4* in Fig. 1) according to the formula (4):

$$f_{S\text{-protein Fisher}}(x) = 7 \cdot 10^5 \cdot \frac{(0.2x)^{4.5}}{(1 + 0.66x)^{13.75}}, \quad (4)$$

while the curve describing the synthesis of antibodies by the long-lived plasma cells (curve *5* in Fig. 1) was approximated by the Erlang distribution (5):

$$f_{S\text{-protein Erlang}}(x) = 1100 \cdot \frac{3.1^{5.1} \cdot (0.11x)^{4.1} \cdot e^{-3.1x}}{4!}. \quad (5)$$

Table 2. Comparison of the experimental data on the content (BAU/ml) of IgG antibodies against SARS-CoV-2 S protein in the blood serum from recovered individuals and the results of calculations using formulas (4) and (5)

Time from the disease onset	S protein [Me (LQ-HQ)]	Calculation according to the Fisher formula (4)	Calculation according to the Erlang formula (5)	Sum of calculations obtained using formulas (4) and (5)	Deviation of the sum of the calculated values from the experimental data, %
1 month	103.3 (73.37-189.1)	89.60	1.23	90.83	12.1%
2 months	456.2 (199.2-1027.1)	439.30	14.96	454.26	0.4%
3 months	787.4 (356.1-1190.2)	687.89	56.07	743.96	5.5%
4 months	819.5 (614.7-1538.3)	718.62	129.69	848.31	–3.5%
6 months	841.8 (614.9-1420.7)	491.38	345.69	837.07	0.6%
12 months	810.9 (504.5-1215.3)	70.82	766.21	837.03	–3.2%
18 months	551.6 (372.5-757.5)	10.98	522.10	533.08	3.4%

When searching for the best values of approximating curve coefficients, we excluded the data corresponding to 6 months from the disease onset. Instead, this data point was used as a test point to avoid model overtraining.

Table 2 compares the experimental data on the levels of anti-S protein IgG antibodies and approximation results obtained using the formulas (4) and (5). In can be seen that the sums of the value calculated using formulas (4) and (5) deviate from the experimentally obtained values by no more than 13% and strictly fall into the calculated LQ-HQ intervals at all time points. When selecting the optimal coefficients for the Fisher and Erlang formulas (3), (4), and (5), in order to assess the quality of the applied models, we calculated the root mean square error (RMSE) and the mean absolute percentage error (MAPE) as the quality metrics. When modeling the changes in the content of anti-N protein IgG antibody with time occurring from the disease onset using the coefficients presented in formula (3), we obtained the minimum values of the quality metrics (RMSE, 16.505; MAPE, 9.408%), indicating a good quality of the proposed model.

The minimum values of the quality metrics (RMSE, 8.949; MAPE, 4.096%) calculated using the coefficients presented in formulas (4) and (5) also indicated a high quality of the proposed model.

The changes in the relative content of IgG subclasses against the N and S proteins are shown in Fig. 2. Interesting, the N protein induced production of all four IgG subclasses (although IgG2 and IgG4 were minor), whereas only the IgG1 and IgG3 subclasses of anti-S protein antibodies were detected, while anti-S protein IgG2 and IgG4 antibodies were absent. The humoral response to both proteins showed the same trend: IgG3 antibodies were gradually replaced by IgG1 antibodies, indicating antibody response maturation, although the rate and completeness of such replacement differed. Thus, IgG1 antibodies represented 54.6 ± 2.7% of all anti-S protein antibodies already one month after the disease onset vs. 35.2 ± 1.1% of anti-N protein IgG1 antibodies at the same time point. Within a year, the fraction of anti-N protein IgG1 antibodies increased to 71.38 ± 3.2% and remained at this level for another 6 months. In the case of the S protein, the content of anti-S protein IgG1 antibodies rapidly increased to 97.4 ± 0.5% 6 months after the disease onset, reached 100% by 12 months in all tested individuals, and remained at this level for at least another 6 months.

Figure 3 shows a comparison between the levels of anti-N protein and anti-S protein IgG antibodies for the four studied groups 3 months after the disease onset (group 1), 3 months after immunization with the second dose of vaccine (groups 2 and 3), and 3 months after the recurrent disease onset (omicron strain) (group 4). The content of antibodies varied in different groups. Thus, in patients who had recovered from COVID-19 once (group 1), the level of anti-N protein

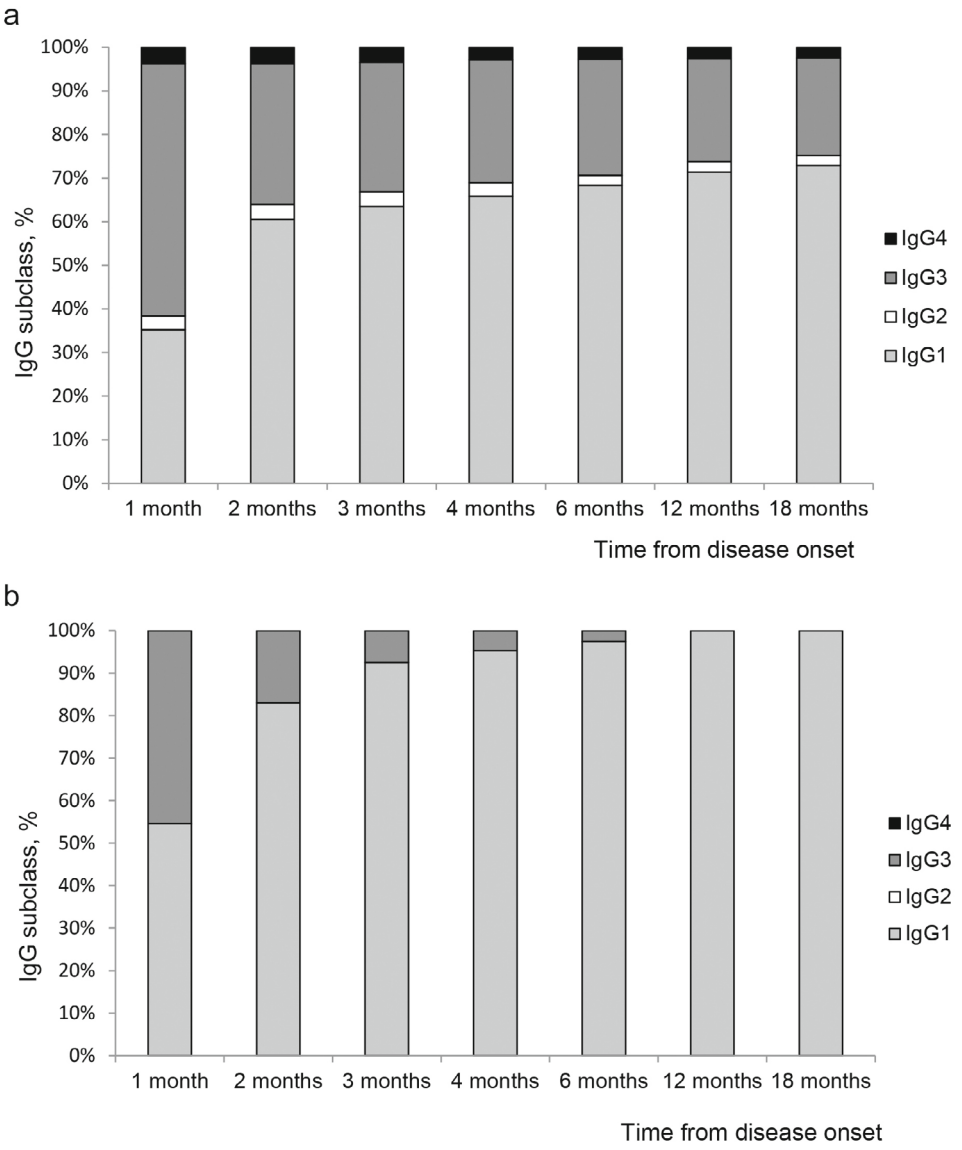

Fig. 2. Changes in the content of different subclasses of IgG antibodies against SARS-CoV-2 antigens. a) N protein. b) S protein.

antibodies varied greatly, but did not significantly exceed the level of anti-S protein antibodies. No anti-N protein antibodies were detected in individuals vaccinated with Sputnik V (group 2), which can be explained by the absence of N protein in this vaccine. In group 3 with the hybrid immunity (vaccination 6-12 months after COVID-19), the content of anti-N protein IgG antibodies was significantly lower (p = 0.018) than the level of antibodies to the S-protein. The content of anti-S protein antibodies in group 3 was significantly higher (p = 0.004) than in group 2. Group 4 with the breakthrough immunity (patients who had COVID-19 twice) demonstrated a significant increase in the levels of both anti-S protein (p = 0.0006) and anti-N protein (p = 0.042) antibodies compared to group 1.

Figure 4 shows a contribution of IgG1 antibodies to the overall IgG response to the N and S proteins. Interestingly, all anti-S protein IgGs in all 4 groups were of the IgG1 subclass. At the same time, the fraction of anti-N protein IgG1 antibodies was 72.8 ± 3.5% in group 1. This subclass of antibodies was completely absent in group 2, since the vaccinated individuals did not form a response to this protein. In group 3, the relative content of IgG1 antibodies did not differ significantly from that in group 1 (75.9 ± 3.8%), which is understandable, since the immune response in these patients had formed during the primary COVID-19 event, while the vaccine used for the following vaccination later did not contain the N protein. In group 4 (patients who had COVID-19 twice), the percentage of IgG1 antibodies reached 99.1 ± 0.3%, which was significantly different from groups 1 and 3 (p < 0.01), indicating that maturation of anti-N protein antibodies continued with the secondary response to the N protein.

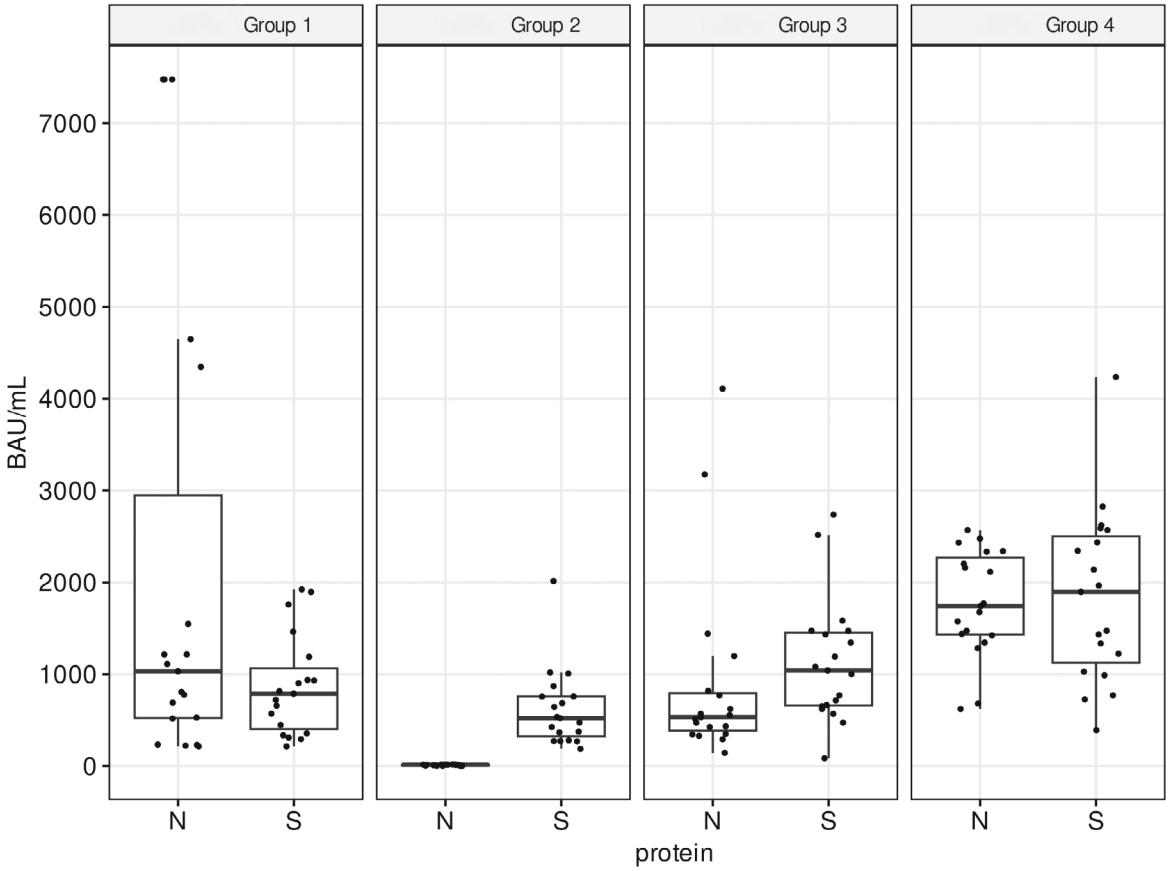

Fig. 3. Comparison of levels of IgG antibodies against N and S proteins in patients recovered from COVID-19 (group 1), individuals vaccinated twice with Sputnik V (group 2), patients who had been ill with COVID-19 and then were vaccinated with Sputnik V (group 3), and patients who had recovered from COVID-19 twice (group 4).

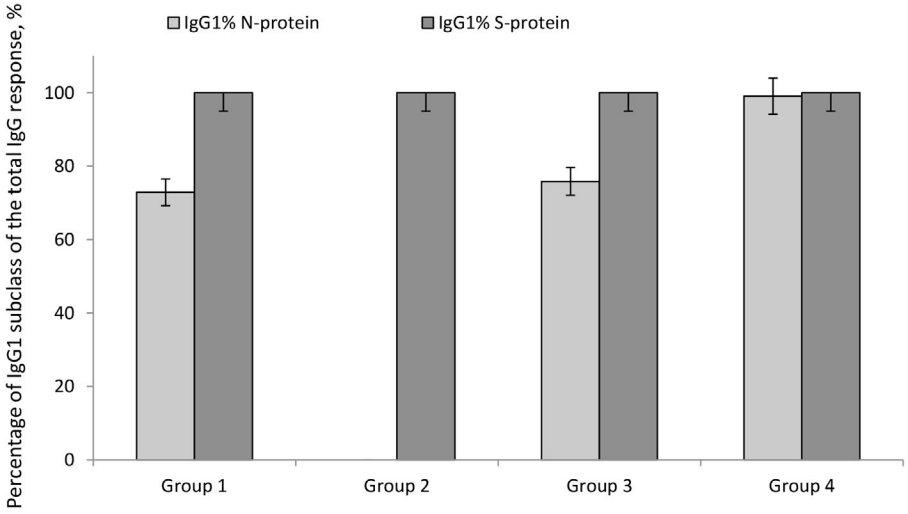

Fig. 4. Contribution of IgG1 antibodies to the overall IgG response to the SARS-CoV-2 N and S proteins.

The results on the cellular immune response to the N and S antigens are presented in Fig. 5. The cellular response to the S protein did not differ significantly between the studied groups, although it was slightly higher in the group with the breakthrough immunity. The cellular response to the N protein in group 3 was significantly lower than in groups 1 and 4 ($p < 0.05$). We expected no cellular response to the N protein in group 2 (individuals vaccinated with Sputnik V), as in the case of humoral immune response. Indeed, we saw no cellular response in 17 people in this group; however, 6 people, who did not have the antibodies

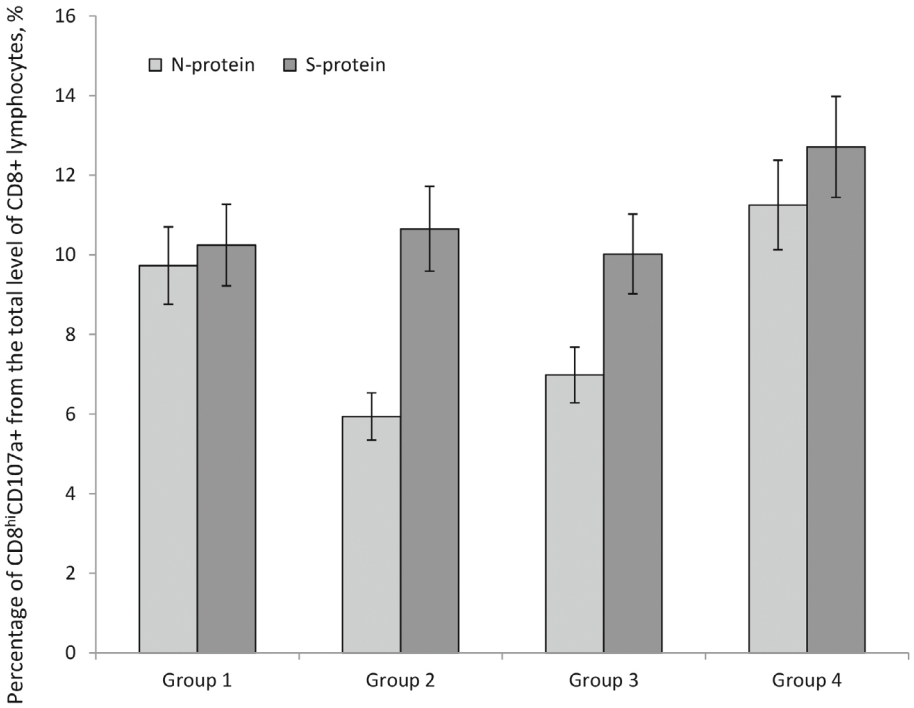

Fig. 5. Cellular immune response to the N and S proteins of SARS-CoV-2.

against the N protein, as well as lacked antibodies to the S protein, prior to the vaccination demonstrated a significant cellular response to the N protein, which resulted in the average response level of 5.94 ± 2.3% in group 2. There was a strong positive correlation between the levels of cellular responses to the N and S proteins (r = 0.937). We also revealed a weak positive correlation between the humoral and cellular responses to the S protein (r = 0.358) and the absence of such correlation for the N protein.

DISCUSSION

We found that in the individuals who had recovered from COVID-19, the concentration of IgG antibodies to the N protein in the blood increased faster than the concentration of anti S-protein antibodies. The content of anti-N antibodies showed a higher and sharper peak and a more rapid decline. Similar results were obtained by other researchers [16]. The curve describing changes in the concentration of anti-N protein antibodies was well approximated by the Fisher distribution (Fig. 1), which is a special case of the Pearson distribution. In the case under consideration, the antibody concentration in the blood was influenced by two independent events: formation of early, short-lived plasma cells that synthesized these antibodies, which was followed by the death of these cells over time, resulting in the decrease in the antibody concentration. The changes in the level of anti-S protein antibodies had a different pattern. The concentration of these antibodies increased more slowly, and instead of a peak, reached a plateau that lasted up to a year, after which the content of the antibodies gradually decreased. Our attempts to approximate this curve with a single function were unsuccessful. We believe that the curve representing changes in the anti-S antibody concentration is a sum of two independent processes. The first process, which is formation of early, short-lived plasma cells synthesizing anti-S protein antibodies, is similar to that observed during formation of anti-N protein antibodies and could be well approximated by the Fisher distribution. The peak formation of the antibodies by the short-lived plasma cells occurred at 3 months after the disease onset for both anti-N protein and anti-S proteins antibodies. The second parts of the curves ran almost parallel to each other, indicating that the respective processes were identical (see Fig. 1). The second event was the formation of long-lived plasma cells that also synthesized anti-S protein antibodies. This second process was more prolonged in time and could be approximated by the Erlang distribution (γ-distribution). This function is also a special case of the Pearson distribution and is applicable to describe the results of two continuous independent events over time, in our case, formation and death of long-lived plasma cells producing anti-S protein antibodies, which was reflected in the concentration of these antibodies in the blood.

It is known that short-lived plasma cells synthesize predominantly IgG3 antibodies, while long-lived

plasma cells produce mainly IgG1 antibodies [17]. Interestingly, the initial response to the N protein represented formation of mostly IgG3 antibodies had been formed in, while the switch to the IgG1 subclass occurred slowly, with the content of IgG1 reaching ~ 70% within 6 months after the disease onset and remaining at this level even after 1.5 years. Similar results were obtained by other researchers [18]. At the same time, in response to the S protein, this switch had occurred much faster; the fraction of IgG1 exceeded 90% within 3 months after the disease onset and then reached 100%. Presumably, these differences are due to the fact that the short-lived plasmacytes dominated in the response to the N protein, while the response to the S protein was accompanied by the formation of long-lived plasmacytes as well. It is also very likely that the differences in the response to the two highly immunogenic proteins of SARS-CoV-2 are associated with the functions of these proteins. Thus, the N protein is located inside the virion and is active at the stage of virus replication; anti-N protein antibodies are not protective [9]. On the contrary, the S protein is located on the virion surface and is responsible for the virus attachment and fusion with the infected host cell; therefore, the antibodies against this protein can block the SARS-CoV-2 infection [19].

If our approximations of changes in the antibody levels are correct, then according to the Erlang function, the level of anti-S protein antibodies should fall slightly below 300 BAU/ml 2 years after the disease onset, decrease to ~40 BAU/ml after 3 years, and drop below 10 BAU/ml (a cut off between the negative and positive levels of these antibodies) after 4 years. Similar dynamics in the antibody levels was observed in patients who suffered from SARS-CoV-1 and MERS viruses [20]. Perhaps. this would have been the case if SARS-CoV-2 had not mutated so often and had been eliminated from the human population. Unfortunately, the reality presents a different picture. SARS-CoV-2 actively mutates; most mutations occur in the S protein, while the N protein remains the most conserved one [21]. Such mutations allow the virus to evade the antibody defense, leading to recurrent illnesses. Also, active vaccination of the population has adjusted the duration of antibody protection against SARS-CoV-2. We studied 4 groups of people who had different histories of contact with SARS-CoV-2. People vaccinated twice with Sputnik V did not differ from those who had recovered from COVID-19 in the anti-S protein antibody level and relative content of IgG subclasses. However, the concentrations of both anti-N protein and anti-S protein antibodies in the individuals who had been ill with COVID-19 twice (at the beginning of the pandemic and again with the Omicron variant) were significantly higher than in people who had been ill once. Interestingly, not only the level of anti-N protein antibodies increased, but these antibodies have become represented almost completely by the IgG1 subclass. This suggests that although the level of anti-N protein antibodies, and therefore the content of plasma cells that synthesize them, were already very low at the time of relapse, memory B cells responded with the secondary immune response to the repeated recognition of the N protein, resulting in additional maturation of anti-N protein antibodies. Anti-S protein antibodies demonstrated a high booster effect in the recurrent disease.

Both S and N proteins induced formation of cellular immune response of $CD8^+$ cytotoxic lymphocytes. It has been shown that T cell respond not only to the structural, but also to the accessory proteins of SARS-CoV-2 [22]. The levels of response to the S protein in the four studied groups did not differ significantly. This indicates that $CD8^+$ lymphocytes are actively involved in the immune response to both the disease and vaccination against COVID-19. Thus, it was shown that pre-existing T cells specific to the SARS-CoV-2 proteins are able to prevent the development of the COVID clinical picture [23]. The level of cellular response to the N-protein in the group with the hybrid immunity (people who had recovered from COVID-19 and were later vaccinated with Sputnik V) was significantly lower than in the group of patients who had recovered from the disease, which could be explained by the absence of N protein in the composition of this vaccine. The discovery of a high cellular immune response to the N protein in 6 people in the vaccinated group was unexpected. However, they did not have anti-N protein antibodies, and before vaccination, no antibodies against the SARS-CoV-2 S protein were detected. We believe that his may be due to a heterologous immune response. It is likely that these people had previously suffered from one of the common cold coronaviruses, which had circulated freely in the human population even before 2019. The N protein is extremely conserved and contains epitopes that can cause the cross-reactivity of the T cell-mediated immunity response among different coronaviruses [13]. Any viral protein can be an antigen for a T cell response and trigger an attack of cytotoxic cells. It is possible that such heterologous immune response to the N protein of the common cold coronaviruses has provided protection in people who had mild or asymptomatic COVID-19. On the other hand, it cannot be excluded that these 6 people suffered from SARS-CoV-2 asymptomatically and without IgG formation, but responded by forming the T cell response, as it has been described before [24].

CONCLUSION

In conclusion, we demonstrated that the humoral immune responses to the S and N proteins of SARS-

CoV-2 could form independently of each other. In our case, the N protein induced formation of predominantly short-lived plasma cells synthesizing IgG antibodies of all four subclasses with a gradual switch from IgG3 to IgG1 (about 70%). The response to the S protein included formation of both short-lived plasmacytes (which formed at the beginning of the response) and long-lived plasmacytes. Short-lived plasma cells respond to the S-protein with the synthesis of IgG1 and IgG3 subclasses, while long-lived plasma cells produced IgG1 antibodies. The changes in the content of antibodies synthesized by the short-lived plasma cells were described by the Fisher distribution, while the Erlang distribution was more suitable to describe the levels of antibodies synthesized by the long-lived plasma cells. The content of antibodies in the groups with the hybrid immunity exceeded the antibody levels in people with the post-vaccination immunity. The content of antibodies in the group with the breakthrough immunity it exceeded that in groups with the post-infectious and post-vaccination immunity. The cellular immunity to the S and N proteins varied depending on the method of immune response induction (vaccination or disease). Importantly, heterologous immune response of $CD8^+$ T cells to the N protein of other coronaviruses may be involved in the immune protection against SARS-CoV-2.

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Ethics declarations. All studies were conducted in accordance with the principles of biomedical ethics as outlined in the 1964 Declaration of Helsinki and its later amendments. Each participant in the study provided a voluntary written informed consent after receiving an explanation of the potential risks and benefits, as well as the nature of the upcoming study. The authors of this work declare that they have no conflicts of interest.

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