Quantitative Assay of SARS-CoV-2 RNA and Level of Proinflammatory Protein Gene Transcripts in Peripheral Blood Leukocytes after a Novel Coronavirus Infection

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Translated from Byulleten’ Eksperimental’noi Biologii i Meditsiny, Vol. 173, No. 6, pp. 726-731, June, 2022
Original article submitted April 20, 2022

The possibility of finding persistent SARS-CoV-2 viral particles in human peripheral blood leukocytes after a novel coronavirus infection was shown. The results of droplet digital PCR showed that 19 of 24 examined subjects had from 4 to 555 copies of the \( Nsp4 \) SARS-CoV-2 gene in 5-6 months after infection. The presence of this transcript in peripheral blood leukocytes was associated with reduced expression of \( FOXP3 \) gene and increased level of \( ROR\gamma \) gene mRNA. The copy number of the \( Nsp4 \) gene negatively correlated with the level of \( FOXP3 \) gene mRNA (\( r=-0.45; \ p=0.028 \)), but showed a positive correlation with the \( DANC\) long non-coding RNA (\( r=0.94; \ p<0.001 \)). In SARS-CoV-2-positive healthy individuals, the level of \( TLR2, NLRP3, \) and \( IL1B \) gene transcripts was higher than in SARS-CoV-2-negative donors. The presence of SARS-CoV-2 in a persistent form is probably associated with impaired immunosuppression and the development of chronic inflammation in apparently healthy volunteers after a new coronavirus infection.

Key Words: SARS-CoV-2; novel coronavirus infection; droplet digital PCR; gene expression; inflammation

A number of viral infections are characterized by alternating latent and lytic (active) phases. For example, gamma-herpesviruses (Epstein–Barr virus, Saimiri herpesvirus in monkeys, and HHV-8 herpes associated with Kaposi's sarcoma in humans) demonstrate alternative life cycles (latent and lytic) upon infection [1]. In the case of a latent or asymptomatic infection, such as herpes, influenza, after a primary infection or between relapses of the disease, the virus is hardly detected or not detected at all in biological fluids. Viruses can be reactivated in host cells when stimulated by various exogenous and/or endogenous factors, and this process is associated with changes in the transcriptional activity of the viral genome or the host genome. Thus, the transition of the Kaposi sarcoma virus HHV-8 from the latent to the lytic phase is the result of transcriptional changes in its genome caused by the replication and transcription activator (RTA) [1].

Since 2019, the main attention of infectious disease specialists has been turned to the study of the emergence and spread of SARS-CoV-2, its epidemiology, diagnosis, clinical course, and treatment of the novel coronavirus disease COVID-19 caused by this virus. COVID-19 is a group of acute infectious diseases characterized by a syndrome of respiratory tract injury, in some cases occurring in a severe form with high mortality, and in some cases asymptomatic [2]. Whether SARS-CoV-2 persists in immune or other body cells after patients with novel coronavirus infection recover remains unknown. There is evidence of long-term persistence of SARS-CoV-2 RNA (up to

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4 months from the onset of the disease) in cells and blood sera, which may be due to the persistence of the pathogen or integration of SARS-CoV-2 DNA into human genome [3].

Virus persistence can affect the host’s immune system and can be accompanied by changes in the profile of inflammatory markers. For instance, in male Norwegian rats, the persistent phase of Hantavirus infection (Seoul virus, SEOV) in tissues supporting increased viral replication (i.e., in the lungs) was associated with reduced production of antiviral (IFNβ, IFNγ) and proinflammatory (IL-1β, IL-6, and TNFα) proteins [4]. It has been shown that hantavirus persistence in rodents is associated with increased activity of regulatory T cells [4]. COVID-19 is often accompanied by lymphopenia with a significant decrease in the level of CD8 (T-cell differentiation marker) and an increase in the CD4/CD8 ratio, which allows predicting the severity of the disease [5]. This leads to an immunosuppressive state and can cause reactivation of other latent viral infections [6]. It is unknown whether the presence of viral particles in the human body after a COVID-19 affects immune parameters and the content of inflammatory markers.

In the case of a latent phase of COVID-19, quantification of residual viruses in clinical specimens is important. Droplet digital PCR allows detecting targets with a concentration of 0.0001% copies, which cannot be detected by other methods.

The aim of the study is to quantify SARS-CoV-2 in peripheral blood leukocytes (PBL) from people recovered from COVID-19 using quantitative real-time PCR (qPCR) and droplet digital PCR (ddPCR) and assess the level of transcripts of genes encoding T-cell differentiation markers and proinflammatory proteins.

**MATERIALS AND METHODS**

Peripheral blood samples for the study were collected from healthy subjects without history of COVID-19 (n=28, mean age 52.0±2.4 years) and subjects recovered from COVID-19 (at least 5 months after recovery; n=24, mean age 50.0±2.7 years). In the latter group, 7 subjects had concomitant diseases: type 2 diabetes mellitus (3 cases) and chronic pancreatitis (4 cases). The material for the study was obtained with the assistance of the V. A. Baranov Republican Hospital (Petrozavodsk, Russia) and Center for Biomedical Research, Karelian Research Centre. Written informed consent to conduct the research study was obtained from all participants (n=52, mean age 51.0±1.8 years). The study was approved by the Ethics Committee of the V. A. Baranov Republican Hospital and was carried out in compliance with the principles of the WMA Declaration of Helsinki.

Among those who were diagnosed with COVID-19 at the time of the disease, the disease manifested as an acute respiratory infection without lung damage in 18 subjects, was asymptomatic in 4 subjects, and was accompanied by lung damage with the development of community-acquired bilateral pneumonia in 2 patients. All participants were negative for SARS-CoV-2 at the time of the study.

To exclude the influence of inflammation, the expression of genes of proinflammatory proteins and immune cell differentiation markers was assayed in PBL of apparently healthy volunteers (n=30, mean age 44.46±3.25 years), who according to the results of our study had a negative (14 people) or positive (16 people) the status of SARS-CoV-2 from the sample for detection of viral RNA by ddPCR. The exclusion criteria were the presence of concomitant immune-inflammatory diseases, infectious diseases during the previous month, tobacco smoking, alcohol abuse, and body mass index ≥28 kg/m².

Total RNA was isolated using PureZole reagent (Bio-Rad) from PBL obtained after treatment of whole blood with 0.86% ammonium chloride solution and centrifugation at 1250 rpm using Liston C 2201 centrifuge. The quality of total RNA was assessed by electrophoresis in 1% agarose gel. The content of total RNA was assessed on a SmartSpecPlus spectrophotometer (Bio-Rad). Synthesis of complementary DNA was synthesized using the MMLV RT kit (Eurogen) on an RNA matrix pretreated with DNase (Synthol). qPCR was performed on a LightCycler amplifier (Roche). PCR mixture (25 µl) contained 5 µl qPCRmix-HS SYBR PCR mix (Eurogen), 10 pmol forward and reverse primers, 30 ng complementary DNA, and 18 µl deionized sterile water. The 18S rRNA was used as a reference gene [7]. The specificity of PCR products was checked by melting curves. Each PCR was performed at least 3 times. The relative level of gene expression was assessed by ΔCt.

ddPCR was performed using Droplet Digital PCR QX200 system (Bio-Rad) and QX200 EvaGreen ddPCR supermix. Primers for PCR were designed using Beacon Designer 5.0 software. The sequence of primers for assessing the level of transcripts of the FOXP3 and RORγt genes was presented elsewhere [8]. The nucleotide sequence of other primers is shown in Table 1.

Statistical data processing was performed using the Statgraphics Centurion XVI 16.1.11 software package. As according to the Shapiro–Wilk test, biochemical parameters did not fit normal distribution. Significance of differences in the level of gene expression was assessed using the Mann–Whitney U test. The results are presented as Me (Q1; Q3). Analysis of variance was carried out using the Kruskal–Wallis H test. The age of the individuals included in the study is
presented as \( M \pm m \). The differences were considered significant at \( p < 0.05 \).

## RESULTS

qPCR detected no copies of the gene sequence encoding the SARS-CoV-2 Nsp4 protein in PBL of the examined subjects, while ddPCR detected copies of this sequence in PBL of 19 donors who had recovered from COVID-19. The number of copies per 1 µl ranged from 4 to 555. Five donors from the group without a history of COVID-19 (SARS-CoV-2 IgG antibodies were not tested) were positive and had 3 to 192 copies/µl. These results indicate the presence of SARS-CoV-2 virus in a latent form in PBL of individuals who have previously had this infection.

The presence of SARS-CoV-2 Nsp4 copies in PBL was accompanied by a decrease in transcriptional activity of the FOXP3 gene and an increase in the expression of the ROR\( \gamma \) gene (Fig. 1). The expression of FOXP3 and alpha subunit of the IL-2 receptor genes (IL2R) is the most important characteristic of T-regulatory lymphocytes (Treg), while the gene encoding transcription factor ROR\( \gamma \) is the most important marker of T-helpers 17 (Th17). Most likely that the presence of virus copies affected the immune cells profile towards a decrease in the content of suppressor Treg cells and an increase in the number of proinflammatory Th17. Similar changes in the CD4/Treg and Th17/Treg cell ratios are observed in some immunoinflammatory diseases, e.g. sarcoidosis [8].

The mRNA level of long non-coding RNA (lncRNA) of DANC\( R \), IL1B, NLRP3, and TLR2 genes in PBL of SARS-CoV-2-positive healthy donors was significantly higher than in subjects with negative status (Table 2). SARS-CoV-2 Nsp4 copy number correlated positively with the level of lncRNA DANC\( R \) transcripts \((r=0.94; p<0.001)\) and negatively correlated with FOXP3 gene expression \((r=-0.45; p=0.028)\). No differences in the content of transcripts of other studied genes were found.

The SARS-CoV-2 S-protein, in addition to the ACE2 protein, also binds to innate immune cell receptors, such as Toll-like receptors (TLR) and NOD-like receptors (NLR) [9]. This can lead to the initiation of signaling aimed at enhancing the expression of genes encoding the components of the NLRP3 inflammasome and the formation of its active complex. It is likely that the presence of viral particles contributes to activation of TLR2 and NLRP3 inflammasome, as evidenced by an increase in the level of mRNA of the gene encoding these receptors, the NLRP3 gene (the

### TABLE 1. Primer Sequence for qPCR

| Gene     | Access number in NCBI | Primer sequence | PCR product size, bp |
|----------|------------------------|-----------------|----------------------|
| Nsp4 SARS-CoV-2 | NC_045512.2 | F: TTTAGCCAGCGTGGTGGTAG R: GGCAACGCAAAAAACCACCTTC | 89 |
| IncRNA DANC\( R \) | NR_024031.2 | F: AGCCGAGGTGGAACAGTACA R: GGACACGTGTTGATCAAG | 156 |
| FOXO1 | NM_002015.4 | F: AACAGGCACACTCTATCATC R: GCACAAATGTTACAGTACACC | 111 |
| FOXO3 | NM_001455.4 | F: TGAGTGAAGAGCAATAGCATAC R: AGCACCTATACGCACATATAAC | 169 |
| IL2RA | NM_000417.3 | F: CACCTCCTCTCTATACCTTCR: TTAGCCCTGTGTCTCTCG | 184 |
| CD8A | NM_001768.7 | F: CACCTCCTCTCTATACCTTCR: TTAGCCCTGTGTCTCTCG | 136 |
| IL1B | NM_000576.3 | F: GATGCTTTATACACTCGGAATG | 139 |
| IL1A | NM_000575.5 | F: GACCAACTCTCCCTCTCTC R: TTAGAATCTCGACAGTCACTG | 176 |
| NLRP3 | NG_007509 | F: GGACATGGACAGCAGCGGT G: TGGTCAGTTAATAAGAGATAACGG | 211 |
| TLR2 | NM_001318787.2 | F: TGATGCTGTGGTGCTTTGG R: AGGTCACTCTGTGATG | 200 |

Note. The source of all presented primers is own design.
cryopyrin gene, the main component of the NLRP3 inflammasome, which is involved in the production of mature form of the proinflammatory cytokine IL-1β and IL1B in SARS-CoV-2 PBL from conditionally healthy donors.

IncRNA are involved in the regulation of inflammatory reactions in response to a bacterial or viral infection. It was found that COVID-19 is also accompanied by changes in the pattern of IncRNA expression. In cells infected with SARS-CoV-2, an increase in the expression of 20 IncRNAs and a decrease in the level of 4 IncRNAs were observed [10]. Enhanced expression of NEAT1 IncRNAs was also recorded in the lung tissues of patients with COVID-19 [10]. IncRNA DANCR is involved in the pathogenesis of inflammatory diseases [11]. This IncRNA can be associated with regulation of FOXP3 transcription factors that, in turn, are involved in maintaining the transcriptional activity of the FOXP3 gene encoding the molecular marker of Treg cells. The decrease in FOXP3 gene expression in SARS-CoV-2-positive donors can be due to DANCR growth expression. In our study, no differences were found in the level of transcripts of the FOXO1 and FOXO3 genes in the compared groups. However, it is well known that DANCR can affect activity and content of FOXP1 and FOXP3. Thus, IncRNA DANCR has been shown to reduce the FOXP1 content in macrophages by participating in the degradation of the ubiquitinated form of this protein [12]. DANCR can also interact with the adapter protein AU-binding factor 1 and increase the content of the FOXP3 protein without affecting its mRNA expression [13].

Thus, copies of SARS-CoV-2 viral particles can probably persist in PBL of patients for at least 5-6 months after recovery from COVID-19. The presence of SARS-CoV-2 Nsp4 transcripts in PBL of healthy individuals probably contributes to impairment of the suppressor properties of T-regulatory cells and enhancement of transcriptional activity of genes encoding proinflammatory proteins.

**TABLE 2.** Expression Level of Genes (relative units) Encoding Markers of T-Cell Differentiation and Proinflammatory Proteins in PBL of Healthy SARS-CoV-2-Negative and SARS-CoV-2-Positive Subjects

| Gene | SARS-CoV-2-negative subjects (n=14) | SARS-CoV-2-positive subjects (n=16) |
|------|-----------------------------------|----------------------------------|
| DANCR | 0.0081 (0.0078; 0.010) | 0.21 (0.14; 0.46)*** |
| FOXO1 | 0.39 (0.17; 0.64) | 0.63 (0.26; 0.88) |
| FOXO3 | 0.14 (0.086; 0.120) | 0.12 (0.06; 0.30) |
| IL2R | 0.029 (0.010; 0.057) | 0.023 (0.009; 0.041) |
| CD8A | 0.009 (0.005; 0.018) | 0.014 (0.008; 0.033) |
| IL1B | 0.10 (0.066; 0.160) | 0.26 (0.055; 0.430)* |
| IL1A | 0.27 (0.03; 0.60) | 0.23 (0.08; 0.30) |
| NLRP3 | 0.043 (0.03; 0.07) | 0.075 (0.05; 0.09)** |
| TLR2 | 0.0025 (0.0060; 0.0064) | 0.0057 (0.0052; 0.0128)*** |

**Note.** *p=0.05, **p=0.02, ***p=0.003, ****p<0.0001.
This work was performed within the framework of research project FMEN-2022-0017 12203100099-1 on scientific equipment of the Common Use Center of the Karelian Research Centre, Russian Academy of Sciences.

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