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Graphical abstract

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Effective virus-neutralizing activities in antisera from the first wave of severe COVID-19 survivors

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Abstract

The pandemic of Coronavirus Disease 19 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become one of the worst public health crises. However, knowledge about the dynamics of antibody responses in COVID-19 patients is still poorly understood. In this study, we performed serological study with serum specimens collected at the acute and the convalescent phases from 104 severe COVID-19 patients who were the first wave of COVID-19 cases in Wuhan, China. Our findings uncovered that neutralizing antibodies to SARS-CoV-2 are persistent at least for more than 6 months in severe COVID-19 patients, despite that immunoglobulin G (IgG) levels against receptor binding domain (RBD) and nucleocapsid protein (N) IgG declined from the acute to the convalescent phase. Moreover, we demonstrate that the level of RBD-IgG is capable of correlating with SARS-CoV-2-neutralizing activities in COVID-19 serum. In summary, our findings identify the magnitude, functionality and longevity of antibody responses in COVID-19 patients, which sheds light on better understanding of humoral immune response to COVID-19, and would be beneficial for developing vaccines.
Introduction

The pandemic of Coronavirus Disease 19 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1-3), has become the worst public health crisis in this century. As of January 4th, 2020, COVID-19 has infected nearly 90 million people and caused over 1.8 million deaths. SARS-CoV-2 is an enveloped, positive-strand RNA virus belonging to the β coronavirus genus and it is the seventh coronavirus that could infect humans so far (4, 5). In terms of clinical manifestations, most of COVID-19 patients have no symptoms or mild symptoms such as cough, headache and myalgia, but the disease course in some patients can progress rapidly to severe and even critical illness (6).

Antibody response plays important roles in host resistance to viral diseases and re-infections, and it is tightly correlated with the convalescent processes of patients (7). Given the emergency and threat caused by the COVID-19 pandemic, it is of high priority to better understand the host antibody responses to COVID-19 patients, particularly the ones with severe symptoms. Up to now, dynamic changes of antibodies against SARS-CoV-2 in COVID-19 patients have been mainly concentrated in asymptomatic patients and patients with mild symptoms (8). However, in patients with severe COVID-19 symptoms, the effectiveness and durability of antibody protection in serum need to be paid more attention after experiencing severe body injuries (9, 10). Besides, this knowledge will be helpful for addressing the most urgent concerns, including re-infection, herd immunity and vaccine efficacy.

The host-derived antibodies to SARS-CoV-2 have been found to target a variety
of viral structural and non-structural proteins (11, 12). Among all the viral antigens, two structural proteins, nucleocapsid (N) protein and spike (S) protein evoke the most common and robust antibody responses found in serum from COVID-19 patients (13-15). N and S proteins are highly immunogenic antigens and frequently used in serological tests for SARS-CoV-2 (16-21). Furthermore, S protein is a large trimeric glycoprotein that contains the receptor binding domain (RBD) (19, 22), which is required for SARS-CoV-2 to bind to angiotensin-converting enzyme-2 receptor, thereby opening the door to entry into target cells (23-25). A number of reports have shown that RBD is the target of the vast majority of neutralizing antibodies in convalescent serum (26-28). Moreover, a recent study identified that the correlation between anti-S and anti-N IgG was moderate, while the anti-RBD and anti-N IgG was better correlated (29).

Notably, the dynamic characteristics of the antibodies with neutralizing activity reflect the protective immune responses in COVID-19 patients and vaccinated population (11, 27). However, little is known about the magnitude, functionality and longevity of neutralizing antibody responses in COVID-19 patients, especially in the severe ones. Herein, we focused on 104 severe COVID-19 patients who were from the first wave of COVID-19 in Wuhan and performed serological tests to measure the RBD-, N- and S-IgG dynamic changes in serum about 6-7 months (median = 195 days, interquartile range [IQR], 188 to 201 days) after disease onset. In addition, the correlation between RBD-IgG levels and neutralizing antibody titers in serum of severe COVID-19 patients was also analyzed.
**Results**

*Clinical characteristics of enrolled 104 severe COVID-19 patients.* We enrolled a cohort of 104 COVID-19 patients who were previously admitted at Wuhan Jinyintan Hospital and diagnosed as severe conditions by the attending doctors according to Chinese Health Commission (6th edition) (30). The disease onset time of these patients was between December 20, 2019 and January 27, 2020, the beginning of the first wave in this pandemic. The clinical and pathological characteristics of these patients are summarized in Supplementary Table S1. It is worth mentioning that all these patients were also enrolled in the clinical trial of lopinavir–ritonavir (31). Serum samples from these patients were collected at the acute phase and the convalescent phase, respectively. The sample collecting time of the acute phase for these patients was 23 days (median, interquartile range [IQR], 20 to 27 days) after the disease onset and that of the convalescent phase was 172 days (median, IQR, 167 to 176 days) after the acute phase sampling. In order to visualize the interval of each sampling points at the acute and the convalescent phase, sampling time-points for each patient were presented in the form of a stacked histogram (Supplemental Figure S1). Additionally, 31 healthy donors were also enrolled in the cohort as controls for comparison.

*Dynamic characteristics of antibodies in severe COVID-19 patients at the acute and the convalescent phases.* We examined the IgG levels against S, RBD and N of SARS-CoV-2 by using ELISA assays, respectively. All the serum samples from 31 healthy donors and 104 severe COVID-19 patients were serially diluted and the area under the
curve (AUC) of S-IgG, RBD-IgG and N-IgG for each sample was measured based on
the OD value at each dilution ratio, respectively. Of all serum samples, one sample
(Patient 15) was used as the internal reference in all the tests for normalization of the
AUC values in all further experiments (Figure 1A). As shown in Figure 1B, the
averaged AUC values of RBD-IgG (24995 ± 9496) and N-IgG (19419 ± 9169) of
COVID-19 patients at the convalescent phase (green lines) were significantly lower
than those at the acute phase (RBD-IgG: 59380 ± 31589; N-IgG: 48889 ± 47288; ****:
P<0.0001) (red lines), while the averaged AUC values of S-IgG at these two time-
points showed no significant difference (acute phase: 25258 ± 24892, convalescent
phase: 21209 ± 9069; \( P = 0.1696 \)). In addition, the AUC values of RBD-, N- and S-
IgG from the convalescent or the acute serum were significantly higher than that from
the healthy serum (*: \( P < 0.05 \); **: \( P < 0.01 \)).

Furthermore, we sought to explore whether the levels of the RBD-, S- and N-IgG
antibodies at the acute and the convalescent phase were related to age or gender. First,
we divided the 104 samples into five groups based on the patients’ age: under 40 years,
41-50 years, 51-60 years, 61-70 years and over 70 years. Our results showed that there
was no significant difference in RBD-, S- or N-IgG levels among the patients from
different age groups either at the acute or the convalescent phase (\( P > 0.05 \), Figure
2A and 2B). Subsequently, we divided these patients into male and female groups.
Similarly, as a result, gender is not a decisive factor affecting the IgG levels at different
phases (\( P > 0.05 \), Figure 3A and 3B).
Effective virus-neutralizing activities in the convalescent serum. We sought to examine whether the convalescent serum still contains the neutralizing activity. To this end, we chose 60 samples from the total 104 samples according to high, medium and low RBD-IgG AUC values, and each group contains twenty samples. In detail, the AUC values of high, medium and low RBD-IgG groups were ranked from the 6th to 20th (AUC: 60900 to 29472), 43rd to 62nd (AUC: 28957 to 21443) and 85th to 104th (AUC: 20497 to 6826) among 104 patients, respectively. There were significant differences in RBD-IgG levels between any two groups (****: \( P < 0.0001 \), Figure 4A). Serum samples of high, medium and low RBD-IgG level groups were then used for examining virus-neutralizing activity. A SARS-CoV-2 strain F13 (BetaCoV/Wuhan/IVDC-HB-envF13/2020) with very high titer and obvious evident cytopathic effect (CPE) when infects Vero-E6 cells were used in the microneutralization assay. The overall titer of neutralizing activity of each sample was measured as the maximum reciprocal dilution at which the serum could inhibit 100 TCID_{50} SARS-CoV-2 completely. As a result, in these 60 samples, the titers of 96.7% (58/60) samples were more than 8, indicating that most of severe patients still contain effective neutralizing activities even more than 6 months after disease onset.

Moreover, we uncovered that the titers of virus-neutralizing antibodies of high, medium and low RBD-IgG groups showed a downtrend with significant differences between each other (*: \( P < 0.05 \), ****: \( P < 0.0001 \), Figure 4B), consistent with the RBD-IgG AUC values. Meanwhile, in order to further determine the correlation between RBD-IgG level and neutralizing antibody titer, correlation analysis was
performed and the results showed that the neutralizing antibody titers were strongly correlated with RBD-IgG AUC values \((r = 0.8349, P < 0.0001, \text{Figure } 4C)\).

**Correlation analysis between RBD-, S- and N-IgG.** Also, we performed the AUC value-based correlation analyses of RBD-, S- and N-IgG at the convalescent phase, and found that RBD-IgG and N-IgG were moderately correlated \((r = 0.5399, P < 0.0001)\) (Figure 5), whereas RBD-IgG and S-IgG \((r = 0.4411, P < 0.0001)\), and N-IgG and S-IgG \((r = 0.1894, P = 0.0542)\) were not correlated. Given that the neutralizing antibody titers were strongly correlated with RBD-IgG levels, our findings indicated that the AUC values of RBD-IgG and N-IgG examined by ELISA were correlated with the titers of SARS-CoV-2-neutralizing antibodies.

**The decreased antibody levels in severe COVID-19 patients at the convalescent phase.**

To further explore the antibody dynamic profiles of severe COVID-19 patients, we compared the AUC values of RBD-IgG, S-IgG and N-IgG of 104 COVID-19 patients at the acute and the convalescent phase and examined the details of the alterations of the antibody levels in each subject. As shown in Figure 6A, the levels of RBD- and N-IgG decreased significantly from the acute to the convalescent sampling points (Paired two-sided Student’s t-tests, ****: \(P < 0.0001\), left and right panel), whereas the levels of S-IgG did not alter significantly at the different time-points \((P = 0.1122, \text{middle panel in Figure } 6A)\). Moreover, the AUC values of RBD-IgG in 91.35% \((95/104)\), S-IgG in 57.69% \((60/104)\) and N-IgG in 93.27% \((97/104)\) of the total patients were found...
Besides, we investigated the details about the percentages of decreased levels of RBD-, S- and N-IgG in 104 patients at the convalescent phase. The degree of antibody decreased in each patient was calculated by the following formula: the degree of decline in antibody (%) = [(AUC value of antibodies at the acute phase) – (AUC value of antibodies at the convalescent phase)] / (AUC value of antibodies at the acute phase).

As a result, the median of the reduction degree was 58.98% (IQR, 48.15 to 68.25%) for RBD-IgG, 15.90% (IQR, 7.83 to 30.91%) for S-IgG and 51.63% (IQR, 31.25 to 66.30%) for N-IgG (Figure 6B-D).
Discussion

The knowledge about the magnitude, functionality and longevity of neutralizing antibody responses in severe COVID-19 patients is poorly understood. In this study, we investigated the virus-neutralizing activities and antibody dynamic profiles of 104 severe patients that were admitted to Wuhan Jinyintan Hospital during the first wave of COVID-19 outbreak in Wuhan, China (32). Our findings provide the evidence that the neutralizing antibodies to SARS-CoV-2 are persistent at least for more than 6 months in severe COVID-19 patients. Moreover, we identified that the level of RBD-IgG is capable of correlating with SARS-CoV-2-neutralizing activity in COVID-19 convalescent serum, consistent with the previous studies (27, 33-35), which provide the possibility that considering RBD-IgG as the targets for constantly monitoring the vaccination effectiveness.

Elucidation of the antibody dynamic profiles in COVID-19 patients not only reveals the prognosis of disease, but also provides experimental basis for practical applications of vaccines. Through the follow-up of early severe COVID-19 patients, we found that although IgG levels of severe COVID-19 patients at the convalescent phase were generally decreased compared to those at the acute phase, the antibodies in serum from more than 95% of patients were still able to neutralize SARS-CoV-2. Our findings are consistent with the previous observations that the neutralizing antibody titers in COVID-19 patients decreased along with the time course after convalescence (8, 36). However, some reports showed that the neutralizing antibodies were consistent with the time frame in COVID-19 patients (11, 37, 38). This discrepancy may be attributed to
the different methods used for examining neutralizing-activity by distinct research groups and the different sampling time-point during the acute phase and/or the convalescent phase, and the cohorts of varied geographical regions. Overall, the current studies reported by others including ours have shown that the serum collected from most of COVID-19 patients at the convalescent phase possesses SARS-CoV-2-neutralizing activity, which supports the notion that the probability of re-infection with SARS-CoV-2 could be largely reduced after six months from the disease onset and the antibody acquisition (39, 40).

Interestingly, as the 104 patients in our study had been enrolled in the trial of lopinavir-ritonavir (31), with 50 in the lopinavir-ritonavir group and 54 in the standard-care group, AUC values were also used to compare the antibody levels in the two groups. Our results showed that no significant difference of IgG levels was found between these two groups at the convalescent phase (Supplemental Figure S2A). Moreover, the percentage of decrease in RBD-IgG level from the acute to the convalescent phase in lopinavir-ritonavir group was lower than that in the standard-care group ($P = 0.0462$, Supplemental Figure S2B), while the percentage of decreased S- and N-IgG levels between these two group showed no difference. Our results suggest that lopinavir-ritonavir may play positive roles on the production or maintenance of antibodies, which are consistent with the previous studies that the counts of B lymphocyte (CD19+) were higher in HIV patients who took lopinavir-ritonavir than those did not (41, 42).

It should be noted that our study has some limitations. First, the limited stock capacity of serum samples is an obstacle to us to further explore the correlation between
RBD-IgG and virus-neutralizing activities in animal protection experiments. Second, more sampling points in a longer period after convalescence should be included for further assessment of RBD-, S- and N-IgG levels and virus-neutralizing activities. In the future, we will keep following up COVID-19 patients at extended time-points to make more accurate and integrated judgments on the robustness and longevity of antibodies and threshold for protection from re-infection.

In summary, our findings identified the magnitude, functionality and longevity of antibody responses in the first wave of COVID-19 patients, which provide valuable data for the research community to better understand COVID-19-associated humoral immunity, and would be beneficial to the efforts for developing vaccines.
Methods

Study design and participants

All 104 subjects in our study had been enrolled in the randomized controlled clinical trial of lopinavir-ritonavir at Jinyintan hospital, Wuhan, China. The disease onset time of patients distributed from December 20, 2019 to January 27, 2020, and the admission time was between January 17, 2020 and March 30, 2020. Diagnosis of SARS-CoV-2 infection was based on clinical diagnostic guideline of Chinese Health Commission (6th edition). Respiratory tract samples of the subjects were positive for nucleic acid of SARS-CoV-2, which were tested by real-time quantitative polymerase-chain-reaction (qRT-PCR) and viral pneumonia of each patient was confirmed with chest imaging by computed tomography (CT). The severity of COVID-19 patients was determined by the attending doctors based on the clinical diagnostic guideline. In addition, demographic data of each subject were collected.

In our study, serum samples of COVID-19 patients were collected during the acute and convalescent phases. The median period from disease onset to acute sampling point was 23 days (IQR, 20-27 days) and the median period from the acute sampling point to the convalescent sampling point was 172 days (IQR, 167-176 days). The collected serum samples were used for subsequent enzyme linked immunosorbent assay (ELISA) of RBD, S and N-IgG and virus-neutralizing activities assay.

Enzyme linked immunosorbent assay

IgG antibodies against RBD, S and N proteins were detected with anti-RBD, S and
N protein Human IgG ELISA Kit (AnyGo Technology Co., Ltd., XG100H8, XG100H7 and XG100H6) according to the manufacturer’s instructions. In short, serum samples of patients were diluted and added into RBD, S or N protein-coated plates, then incubated for 30 min. After washed with 1×PBST 4 times, horseradish peroxidase conjugated anti-human IgG antibodies were added and incubated for 15 min at room temperature. After another more rounds of washes, tetramethylbenzidine substrates were added and incubated for 5-10 min before termination. Then the plates were read at 450 nm and 630 nm with F50 infiniteinfinite® (TECAN).

**Microneutralization assay**

Vero E6 cells were seeded at 1×10^5 per well in a 96-well culture plate at 37 °C for 24 h before use. Serial 2-fold dilutions of 50 µl of serum were prepared in a 96-well tissue culture plate in DMEM medium. An equal volume of SARS-CoV-2 working stock containing 200 TCID_{50} was added, and the antibody-virus mixture was incubated at 37 °C for 1 h. Serum from healthy donors were used as negative controls. The antibody-virus mixture was then added into a 96-well microtiter plate containing equal volume of confluent Vero E6 cells with 8 repeats and incubated at 37 °C in CO₂ incubator for 3 days. Cells infected with 100 TCID_{50} of SARS-CoV-2 and cells without infection were used as positive and uninfected controls, respectively. Cytopathic effect (CPE) in each well was observed daily and recorded on day 3 post infection. A virus back-titration was performed to assess the correct virus titer used in each experiment.
**Statistical analysis**

All consecutive data are described as the medians (IQRs) or the means ± SD, and categorical data are described as numbers (%). Unpaired two-sided Student’s t-tests were used to compare two unpaired groups of variables. Paired two-sided Student’s t-tests were used to compare the significance of paired samples. One-way analysis of variance (ANOVA), Student-Newman-Keuls multiple comparisons test was performed to test differences of continuous variables among multiple groups. A P value less than 0.05 was considered significant (*: P < 0.05; **: P < 0.01; ***: P < 0.001; ****: P < 0.0001). The Pearson correlation coefficient (r) and the probability P value were calculated using GraphPad Prism, version 8.

**Study approval**

This study conformed to the 1975 Declaration of Helsinki guidelines and was approved by the Ethics Committees of Wuhan Jinyintan Hospital (KY-2020-83.01). Written informed consents were obtained from all involved patients.

**Author Contributions**

YW and XZ conceived the study. DYZ, GZW, YH, PPL and YQ designed the experiments. YH, PPL, JZ, YL, XJH, QYY, RH, XYW, HS, PCY, MJY and WJL performed the experiments. YH, PPL, YQ, JZ and KP analyzed data and interpreted the results. The majority of the manuscript was written by YH, PPL and YQ, with some help from YW, XZ, DYZ and GZW. All authors approved the final version of the
manuscript. The order of the co–first authors was determined by their relative contribution to this study.

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Competing interests

The authors have declared that no conflict of interest exists.

Reference.


Graphical Abstract

Figures.

Figure 1
Figure 1. Calculation of antibodies in severe COVID-19 patients at the acute and convalescent phases. (A) ELISAs measuring antiserum reactivity to RBD, S and N proteins were showed; Optical density units at 450 nm (OD, Y axis) and reciprocal plasma dilutions (X axis). The red lines represent the antibody dilution curves for the 104 severe COVID-19 patients at the acute phase, the green lines are antibody dilution curves for these patients at the convalescent phase, and the black curves indicate the antibody dilution in the 31 healthy subjects. (B) RBD-, S- and N-IgG of severe patients during acute and convalescent phases were shown, and the results of healthy subjects by the same analysis were used as the control group. All kinds of IgGs were calculated according to the normalized AUC values in (A). The p value between any two groups was calculated by Student-Newman-Keuls multiple comparisons test, ANOVA, **** P < 0.0001, *** P < 0.001, ** P < 0.01, * P < 0.05.

Figure 2

Figure 2. Antibody levels of different age groups in severe COVID-19 patients at
the acute and the convalescent phases. The 104 severe COVID-19 patients were divided into five groups by age (under 40 years, 41-50 years, 51-60 years, 61-70 years and over 70 years). RBD-, S- and N-IgG levels of each group at the acute (A) and the convalescent (B) phases were shown according to normalized AUC values. The $p$ value among different groups was calculated by ANOVA analysis, $P > 0.05$, no significant difference.

**Figure 3**

**Figure 3. Antibody levels of different gender groups in severe COVID-19 patients at the acute and the convalescent phases.** The 104 patients were divided into two groups by gender. Graphs showed RBD-, S- and N-IgG levels of each group at the acute (A) and the convalescent (B) phase by normalized AUC values. The $p$ value between any two groups was calculated by unpaired two-sided Student’s t-test, $P > 0.05$, no significant difference.
Figure 4. Analysis of correlation between RBD-IgG and neutralizing antibody titers. (A) According to the normalized AUC values, a total of 60 serum samples were divided into three groups based on the RBD-IgG levels of high, medium and low. (B) Neutralizing antibody titers by using the authentic SARS-CoV-2 were detected for serum samples indicated by (A). The $p$ value between any two groups in (A) and (B) was calculated by Student-Newman-Keuls multiple comparisons test, ANOVA, **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. (C) Correlation analysis between normalized AUC values of RBD-IgG and neutralizing antibody titers in the 60 serum samples from (A) and (B). There was a strong correlation between neutralizing antibody titer and RBD-IgG ($P < 0.0001$, $r = 0.8349$). Pearson correlation coefficient was used to determine the $r$ value of the correlation between the two groups.
Figure 5. Correlation analysis among RBD-, S- and N-IgG in severe COVID-19 patients at the convalescent phase. According to normalized AUC values, correlation analysis of RBD-, S- and N-IgG were performed. RBD-IgG and N-IgG were moderately correlated ($r = 0.5399$, $P < 0.0001$, A), whereas RBD-IgG and S-IgG ($r = 0.4411$, $P < 0.0001$, C), and N-IgG and S-IgG ($r = 0.1894$, $P = 0.0542$, B) were not correlated. Pearson correlation coefficient was used to determine the $r$ value of the correlation between any two groups.
Figure 6. Analysis of the decreased tendency of IgG levels in severe COVID-19 patients. (A) Dynamic changes of normalized AUC values of RBD-, S- and N-IgG levels during acute and convalescent phases. The $p$ value was calculated by paired two-sided Student’s $t$-tests. **** $P < 0.0001$, $P > 0.05$, no significant difference. (B) The left panel shows the number of patients with increased or decreased antibodies in the 104 patients, the middle panel shows the decreased percentage of antibodies in patients, the distribution of percentage of antibody reduction for these patients is shown in the right panel.