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Immune evasion and chronological decrease in titer of neutralizing antibody against SARS-CoV-2 and its variants of concerns in COVID-19 patients

Masaru Takeshita, Naoshi Nishina, Saya Moriyama, Yoshimasa Takahashi, Makoto Ishii, Hideyuki Saya, Yasushi Kondo, Yuko Kaneko, Katsuya Suzuki, Koichi Fukunaga, Tsutomu Takeuchi, for the Keio Donner Project

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ABSTRACT

Many variants of SARS-CoV-2 have emerged, and decreased neutralizing antibodies after vaccination and breakthrough infections have become a problem. The importance of monitoring titers of neutralizing antibodies is getting higher. We enrolled 146 COVID-19 patients, who were thought to be infected with Wuhan-hu-1 or Delta4G strains, and examined the time course of neutralizing titers against six concerning strains (Wuhan-hu-1, Alpha, Beta, Gamma, Kappa, and Delta) using newly developed ELISA. The acquisition of neutralizing titer was positively associated with disease severity. Immune evasions were observed approximately 20 to 30% for Alpha, Kappa, and Delta variant, and 40 to 45% for Beta and Gamma variant. The titers against all strains decreased over time, and interestingly, while titers against Wuhan-hu-1 decreased by 23%, those to Delta variant decreased by 70%. Our simple, cost-effective, and non-hazardous system will be applicable to process numerous samples, such as monitoring titers against prevalent strains after infection or vaccination.

1. Introduction

SARS-CoV-2 acquires mutations as the infection spreads. In particular, mutations in the receptor binding domain (RBD) region of the spike protein, which is used by the virus to enter cells, may cause changes in infectivity and in the reactivity of neutralizing antibodies [1–3]. Even in the early stage of the pandemic, the D614G mutation increased and replaced the previous strain within a few months; however, since this mutation was outside the RBD, it did not have a serious impact on the titers of neutralizing antibodies, and increased transmissibility is thought to be the cause of its spread [3,4].

Three variants have been reported from 2020 and designated as VOCs by the World Health Organization (WHO): B.1.1.7 (Alpha variant), which first emerged in England; B.1.351 (Beta variant), which first emerged in South Africa; and P.1 (Gamma variant), which first emerged in Brazil. The Alpha variant is reported to have little effect on neutralizing titer [5–7] or only a mild decrease of 22% [8]. In contrast, strong immune evasion was reported for the Beta and Gamma variants. The Beta variant shows an 8- to 13-fold reduction in convalescent plasma, and a 7- to 9-fold or 65% reduction in vaccinated serum [6,7,9]. The Gamma variant shows a 3- to 5-fold reduction in convalescent and vaccinated plasma/serum [7,10,11]. Even therapeutic monoclonal antibodies have been reported to have reduced or lost neutralizing ability against variants with the E484K mutation [7,9,12].

In April and May 2021, there was a rapid increase in the number of patients in India, mainly due to the B.1.617 strain [13]. This lineage has three main subtypes: B.1.617.1 (Kappa variant) and B.1.617.3, characterized by L452R and E484Q mutation in RBD, and B.1.617.2 (Delta variant), characterized by L452R and T478K in RBD. Due to their high transmissibility, these variants have spread to 96 countries as of the end

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of June, and the WHO has classified the Kappa variant and the Delta variant as a variant of interest (VOI) and a variant of concern (VOC), respectively [13]. These variants have also been reported to escape from humoral immunity, with the Kappa variant showing a 2- to 7-fold decrease in recovered sera and a 3- to 7-fold decrease in post-vaccination sera [14-16], and the Delta variant showing a 6-fold decrease in recovered sera and a 5- to 8-fold decrease in post-vaccination neutralizing titer [16,17]. Immun evas ion also shows up in the vaccine’s effectiveness: 89.5 to 93.7% for the Alpha variant, 75.0% for the Beta variant, and 88.0% for the Delta variant compared to 95% for the original Wuhan-hu-1 strain [18,19].

In Japan, variants with the D614G mutation were initially the majority, and VOC and VOI strains were not detected until the end of 2020. Three VOC strains, Alpha, Beta, and Gamma variants, were first detected in airport quarantine on December 25, 2020, December 28, 2020, and January 6, 2021, respectively. A sample taken on January 22 was the first time that the Alpha variant was detected in Japan from a person with no history of travel abroad [20]. Therefore, by the end of 2020, all of the three VOC strains, Alpha, Beta, and Gamma variants, were first detected in Japan. Therefore, by the end of 2020, all of the three VOC strains, Alpha, Beta, and Gamma variants, were first detected in Japan. Therefore, by the end of 2020, the Delta variant became dominant in early 2021 and the Delta variant has been dominant since June/July [21]. Therefore, it seems important to examine the titers of neutralizing antibodies against these VOCs, after infection with non-mutated strains or vaccination. It is also important to examine the time-dependent attenuation of the titers, as previously reported [22].

We have previously measured the titer of neutralizing antibodies from convalescent patients using magnet beads [23]. In this study, we developed this system as a competitive enzyme-linked immunosorbent assay (ELISA), and validated it using commercially available ELISA kits and sera that had been previously evaluated by an authentic virus neutralization assay [23]. In addition to the ELISA using RBD of the original Wuhan-hu-1 strain-derived sequences, we also produced ELISA using RBD derived from five different variants (Alpha, Beta, Gamma, Kappa, and Delta). Here, we measured the titers of neutralizing antibodies against each variant in the sera of patients infected by 2020, who are assumed to have been affected by the original strain, and in the sera of vaccinated volunteers. The attenuation of the titers of neutralizing antibodies was also examined using serum samples collected over the course of time.

2. Materials and methods

2.1. Clinical samples

We recruited patients who had COVID-19, diagnosed by approved reverse transcriptase polymerase chain reaction (RT-PCR) tests for SARS-CoV-2 using swabs from the nose or saliva, and hospitalized at Keio University Hospital between April and December 2020. Serum samples from patients were collected at outpatient visits over a period of 6 months to 1 year. We also collected the serum from healthy volunteers 1.5 to 2 months after their second Pfizer/BioNTech vaccination.

The following parameters were collected from medical charts: signs and symptoms; neutrophil and lymphocyte counts; serum parameters of lactate dehydrogenase (LD), C-reactive protein (CRP), ferritin, D-dimer, sialylated carbohydrate antigen KL-6, estimated glomerular filtration rate (eGFR); and medication history. Pneumonia was diagnosed based on lung computed tomography (CT). The titers of neutralizing antibodies against 5-9 authentic virus for some sera were derived from the previous study [23]. This study was approved by the Ethics Committee of Keio University School of Medicine and conducted in compliance with the tenets of the Declaration of Helsinki. Informed consent was obtained from all participating individuals.

2.2. Production of recombinant ACE2 and SARS-CoV-2 proteins

Recombinant soluble ACE2 (1-708 AA) and RBD (Spike 319-541AA) were produced as previously described with minor modifications [23]. Briefly, the extracellular domain of ACE2 was inserted into the pcDNA3.4 expression vector (Thermo Fisher Scientific, MA, USA) with a polyhistidine tag at the N-terminus. The RBD of the spike was inserted into pcDNA3.4 with an SBP tag at the C-terminus. The RBD vectors of Alpha, Beta, Gamma, Kappa, and Delta variants were produced by PCR using mutated primers. The recombinant RBD and ACE2 were produced using the Exp293 Expression System (Thermo Fisher Scientific) according to manufacturer’s instructions. The supernatants of RBD were concentrated and buffer exchanged into phosphate-buffered saline (PBS) using Amicon Ultra filters (Merck, Darmstadt, Germany) and incubated with Sepharose 4B beads (Merck) overnight at 4 °C with shaking for preclear. The cleared supernatants were then incubated with Streptavidin Sepharose High Performance beads (Cytiva, Tokyo, Japan) over night at 4 °C with shaking. The beads were washed 5 times with PBS, and proteins were eluted with 1 × Buffer BXT (IBA, Gottingen, Germany).

The supernatants of His-tagged ACE2 were directly purified using His gravitrap TALON column (Cytiva) according to manufacturer’s instruction. The eluted proteins were buffer exchanged into PBS using Amicon Ultra filters, and stored at 4 °C. The purities of recombinant proteins were determined by SDS-PAGE and Coomassie Brilliant Blue (CBB) staining using a 12.5% Supersep precast gel and Quick CBB Plus (FUJIIFILM Wako, Osaka, Japan), and the concentrations were determined by a BCA Protein Assay Kit (Thermo). The results of CBB staining and mutation information are shown in Supplementary Fig. 1.

2.3. ELISA

We designed the in-house ELISA system that measures the extent to which serum prevents a certain amount of RBD from binding to a certain amount of ACE2, by mimicking the process of infection in which serum prevents a certain amount of Spike protein on virus from binding to a certain amount of ACE2 expressing cell to be infected (Supplementary Fig. 2). The measurement protocol is as follows: 96-well plates (Thermo, 442,404) were coated with 10 μg/ml of Wuhan-hu-1 RBD or mutated RBD in PBS overnight. Plates were washed with PBS with 0.05% Tween-20 (PBS-T), and blocked with blocking buffer (Bethyl Laboratories, TX, USA) for 30 min at room temperature (RT). After washing, plates were incubated with 1:10 diluted serum in diluent buffer (blocking buffer with 0.05% Tween-20) for 1 h at RT. After washing, plates were incubated with 5 μg/ml of His-tagged ACE2 in diluent buffer for 1 h, washed, and incubated with horseradish peroxidase (HRP)-conjugated anti-Histag antibody (MBL) at 1:3000 dilution for 1 h. After final washing, plates were incubated with TMB Substrate Set (BioLegend, CA, USA) for 15 min. Reactions were stopped with H2SO4 and the optical density at 450 nm (OD450) was measured.

The inhibition rate was calculated as follows: Inhibition rate = 1–(OD450 of sample) / (OD450 of blank). To show the results as comparable linear values, inhibition rates were converted to titers of neutralizing antibody based on a standard curve as follows: the sample with the highest titer of neutralizing antibody was defined as the standard serum, its titer against the Wuhan-hu-1 strain was set to 100 U/ml, and the standard curve was obtained from the dilution series of the standard serum. The conversion formula is as follows; [Inhibition rate (%)] = (a – d) / (1 + ([neutralization titer (U/ml)] / c) ^ b) + d; a = 4.596, b = 2.711162, c = 37.18781, d = 99.51899. The limits of quantitation were 2.9, 6.1, 0.8, 2.0, 12.4, and 1.4 U/ml for Wuhan-hu-1, Alpha, Beta, Gamma, Kappa, and Delta variant respectively, calculated by the formula as follows; [Limit of quantitation] = 10 × [standard deviation of the blank] / [the slope of the calibration curve at low concentration].

The serum titers of neutralizing antibody were also measured by commercially available kit, a SARS-CoV-2 Neutralization Antibody Detection Kit (MBL, Tokyo, Japan) at 1:10 dilution, and the inhibition rate between RBD and ACE2 was calculated according to manufacturer’s instructions.
The correlation between the neutralizing titer from the in-house ELISA and the authentic virus neutralizing titer are shown. The samples below the detection limit (1:5) were calculated as 2.5. Spearman’s rank correlation coefficient. 

### Table 1
Clinical characteristics of the patients.

| Disease severity | Asymptomatic (n = 19) | Mild (n = 42) | Moderate (n = 74) | Severe (n = 5) | Critical (n = 6) | p value |
|------------------|-----------------------|--------------|------------------|--------------|----------------|---------|
| Sex (male)       | 11 (58)               | 20 (48)      | 51 (69)          | 4 (80)       | 6 (100)        | 0.045   |
| Age (year)       | 31 (25-40)            | 30 (26-44)   | 54 (37-61)       | 60 (53-65)   | 61 (54-71)     | <0.0001 |
| Smoking: never / ex / current | 15 / 2 / 2 | 33 / 4 / 5 | 41 / 24 / 9 | 0 / 4 / 1 | 0 / 6 / 0 | <0.0001 |
| Days between first positive PCR test and first serum collection | 51 (32-69) | 49 (41-56) | 53 (47-62) | 51 (31-63) | 62 (52-81) | 0.187   |
| Previous coexisting disease | 10 (53) | 23 (55) | 52 (70) | 5 (100) | 5 (83) | 0.103   |

### Table 2
Clinical characteristics of the vaccinated volunteers.

|                     | Vaccinated volunteers (n = 10) |
|---------------------|--------------------------------|
| Sex (male)          | 8 (80)                         |
| Age (year)          | 36 (33-49)                     |
| Days after second vaccination | 53 (52-54)                |

#### 2.4. Authentic virus neutralization assay

The authentic virus neutralization assay using SARS-CoV-2 virus (hCoV19/Japan/TY-WK-521/2020) and the variant strains (Alpha; hCoV19/Japan/QHN002/2020, Beta; hCoV19/Japan/TY8-612-P1/2021, Gamma; hCoV19/Japan/TY7-503/2021, and Delta; hCoV19/Japan/TY11-927-P1/2021) were performed as previously described [24]. Briefly, a mixture of 100 TCID50 virus and serially diluted antibodies (2-fold serial dilutions starting at 1:5 dilution) was incubated at 37 °C for 1 h before being placed on VeroE6/TMPRSS2 cells (JCRB Cell Bank, Osaka, Japan) seeded in 96-well flat-bottom plates. After cultivating for five days at 37 °C, cells were fixed with 20% formalin (Fujifilm Wako), and stained with crystal violet solution (Merck). Each sample was assayed in two wells and the lower cut-off dilution index with >50% cytopathic effect was presented as neutralization titer.

#### 2.5. Statistics

Continuous data are presented as the median and interquartile range (IQR) or as a number with the percentage value, as appropriate. The chi-square test and Fisher’s exact test were used for categorical variables. Wilcoxon’s test was used for continuous variables. Spearman’s rank correlation coefficient was used for correlation analysis.
A squared test was used to examine the categorical variables. The Wilcoxon rank sum test and Wilcoxon signed rank test were used to examine the continuous variables with and without correspondence, respectively. The Steel’s multiple comparison test was used for comparison between multiple groups. Correlations between two continuous variables were analyzed using Spearman’s rank correlation coefficient. A model for the authentic virus neutralizing titer was made using multiple linear regression analysis. p-values < 0.05 were considered to be statistically significant. The variables that were associated with the neutralizing titer were entered into the model using forward selection with a threshold p-value of 0.05. All statistical analyses were performed with JMP 15 (SAS Institute, NC, USA).

Table 3
Multivariate analysis of clinical symptoms and neutralizing titer.

| Characteristics                  | Coefficient (95% confidence interval) | t-value | p-value |
|----------------------------------|---------------------------------------|---------|---------|
| Disease severity > Moderate      | 3.55 (0.83–6.28)                      | 2.57    | 0.0111  |
| Highest CRP level (mg/dl)        | 0.74 (0.12–1.37)                      | 2.35    | 0.0203  |
| Highest LD level (U/l)           | 0.08 (0.05–0.12)                      | 4.29    | <0.0001 |
3. Results

3.1. Verification of in-house ELISA

We collected 220 sera from 146 patients in the convalescent phase of COVID-19, of which, 42 samples had been previously evaluated by authentic virus neutralization assay [23], and sera from 10 vaccinated volunteers. The characteristics of participants and vaccinated volunteers are shown in Tables 1 and 2. First, in order to confirm the performance of our in-house ELISA, we examined whether the results of the in-house ELISA correlated with the results of the authentic virus neutralization assay and commercially available ELISA kit for SARS-CoV-2 neutralizing antibody. As shown in Fig. 1, the titers of neutralizing antibodies measured by in-house ELISA were well correlated with those measured by authentic virus neutralization assays and inhibition rates by commercially available ELISA. The titers by authentic virus neutralizing assays were also correlated with the inhibition rates by commercially available ELISA. Therefore, in-house ELISA was confirmed to have performance equivalent to that of the commercial kit.

3.2. Correlations between neutralizing titer and clinical parameters

Next, we compared the neutralizing titers of the first serum samples, collected a median of 49 to 62 days after the first positive PCR test, with various clinical parameters. As shown in Fig. 2, median titers of neutralizing antibodies positively correlated with disease severity, and patients with severe or critical disease had more than twofold the titers of neutralizing antibodies than those with mild or moderate disease. The titers of neutralizing antibodies also correlated with age, disease symptoms (including fever, upper and lower respiratory symptoms, and pneumonia), and serum parameters (including lowest lymphocyte count, CRP, LD, ferritin, and D-dimer). The titers of neutralizing antibodies in post-vaccinated individuals were similar to those in patients with severe or critical disease severity. Next, we performed a multivariate analysis using the clinical parameters that correlated with the titers of neutralizing antibodies. As shown in Table 3, disease severity, highest CRP level, and highest LD level were identified as the independent variables correlating with the titers of neutralizing antibodies, consistent with the previous studies [22,23,25].

3.3. Titers of neutralizing antibodies against mutated RBD

Next, we measured the titers of neutralizing antibodies against variant strains using the in-house ELISA system. In order to compare the effect of mutations only, mutated RBD protein production and ELISA procedures were performed in exactly the same manner, except that mutated vectors were used. First, we examined whether these ELISAs using mutated RBD correlated with the results of the authentic virus neutralization assay. We selected 10 sera from representative patients and confirmed that in-house ELISA using RBD with mutation correlated with the authentic virus neutralization titer as well as with Wuhan-hu-1 (Fig. 3A). In overlaying these correlations (Fig. 3B), the strains other than Beta showed similar correlations to those of Wuhan, although some variation was observed in Beta variant, suggesting that the neutralizing titers measured by in-house ELISA were comparable among strains with a small variation.

Because variant strains such as Alpha variant have been reported in Japan since January 2021 [20], and because this study used samples from patients who had been infected by COVID-19 before that time, all patients were thought to be infected with the Wuhan-hu-1 strain or the strain with D614G. Fig. 4A shows that the serum titers of neutralizing antibodies against the Wuhan-hu-1 strain were generally correlated with those against each variant strain. The distribution of some of the plots is skewed to the lower right, indicating that the neutralizing titers against the variant strain are lower than those against Wuhan-hu-1 strain. In particular, the Beta and Gamma variants, which commonly have the E484K mutation, are shifted more than the red line in the center.

We further stratified the samples by disease severity, and compared the titers. As shown in Fig. 4B, there was little difference in asymptomatic and mild cases with low titers, but especially in the group of severe and critical cases, there was an approximately 20 to 30% decrease in the Alpha, Kappa, and Delta variants, and an approximately 40 to 50% decrease in the Beta and Gamma variants. This attenuation of the titers of the neutralizing antibodies was also observed in post-vaccinated individuals to the same extent as in the severe and critical group.

3.4. Longitudinal changes in the titers of neutralizing antibodies

Finally, we examine the changes in the titers of neutralizing antibodies over time. Because it is difficult to calculate the rate of decline for samples with a low neutralizing titer from the start, we selected patients with moderate to critical disease severity, and calculated the decline rate of the titers using the results of the first and second test (median, 47.5 and 112 days after first positive PCR, respectively). As shown in Fig. 5A, the serum neutralization titer against Wuhan-hu-1 strain decreased by about 23% in two months, and interestingly, the titer reduction against
Alpha, Gamma, and especially Delta variants was significantly faster than that against Wuhan-hu-1 strain. Fig. 5B shows the titers of cases that could be measured three times or more. Although the number of patients was limited, the patients with higher neutralization titers tended to have a faster decrease in their titers up to 3 to 4 months after infection than later.

4. Discussion

In this study, we established a highly versatile ELISA system for measuring the titers of neutralizing antibodies that correlate well with the results of authentic virus neutralization assay. In addition, the titers of neutralizing antibodies against the major variants were also examined in a comparable manner using the same protocol and mutation-introduced RBDs, and confirmed the correlation with the authentic virus neutralization assay using variant strains. The results showed that the titers of neutralizing antibodies correlated well with disease severity in COVID-19 patients. In addition, the vaccination-induced antibody titers were comparable to the neutralizing titers in severe and critical COVID-19 patients. Immune escape was observed in VOCs and the Kappa variant, and the titer decreased over time.

As for the immune evasion of variant strains, it is reported that the
neutralizing titers decreased 6.3-fold in the Beta variant, 4.3-fold in the Kappa variant, and 5.1-fold in the Delta variant using a pseudovirus assay system [16]. The immune evasion of Kappa and Delta appears to be weaker than that of Beta and Gamma, according to other reports [14,17]. Some of the neutralizing antibodies bind to N-terminal domain (NTD) of Spike protein, and the neutralizing ability of these antibodies is reduced by mutation of NTD such as in Alpha variant [26]. Even though only antibodies against RBD were examined in our assay, the results correlated with the authentic virus neutralization assay. This is probably due to the major epitope of the neutralizing antibody existing in the RBD [27]. The ranges of the reduction of the neutralizing titers were approximately 20 to 30% for the Alpha, Kappa, and Delta variants, and 40 to 50% for the Beta and Gamma variants in severe and critical cases and vaccinated samples in this study. Although there were some differences in the reduction rate depending on the assay system, the results of this method were correlated with those of the authentic virus neutralization assay, and the trend for each strain was similar.

The titers of neutralizing antibodies from COVID-19 patients decreased by about 23% in two months. The serum half-life of the neutralizing titers after vaccination was reported to be 14.7 weeks [22], and the results of both studies were similar. In Fig. 5B, the results of the third sample indicate that the decrease in the neutralizing titers appears to be moderate, although the sample size is small. It was reported that the titers of neutralizing antibodies were maintained throughout a 13-month observation [28], and the long-term trend of the titers is thus an issue to be addressed. In Fig. 5A, the decrease in the neutralization titers was faster against Alpha, Gamma, and Delta variants than against Wuhan-hu-1. In particular, the titer against Delta variant was reduced by a median of 70%. This could have contributed to the spread of infection and the replacement of existing strains. Therefore, it is important to establish a cutoff value of the neutralization titers against each strain that can protect against re-infection or breakthrough infection. Recently, breakthrough infections after vaccination have been reported to be linked to a lower antibody titer [29], and the cutoff value will become important as an indicator for the additional vaccination of healthcare workers.

There are several limitations in this study: first, because most of the participants were infected by the Wuhan-hu-1 strain with or without D614G mutation, we cannot assess immune evasion among other strains, such as the neutralization titers of the Delta variant after infection with the Alpha variant. Second, because vaccination in Japan started on a full scale in April/May 2021, the number of vaccinated samples was small and the changes in the time-series could not be evaluated. Third, the standard curve of Wuhan-hu-1 was used for other strains to calculate the neutralization titer from the inhibition rate, in order to evaluate neutralizing titer including the effects of immune evasion, in the absence of a golden standard value across variant strains. Although it may contain errors in the calculated values of variant strains, we performed a neutralization assays using variant strains and confirmed the correlation between calculated value and neutralization titers by general neutralization method (Fig. 3B). Only the Beta variant differed slightly from the other strains, which may have been due to impurities in the purified RBD (Supplementary Fig. 1A), and this may lead to the variation in the neutralization titer of Beta variant.

5. Conclusions

The authentic virus and pseudo-virus neutralization assay may reflect a more native infection process than the competitive ELISA used in this study, however, our cell- and virus-free method, as well as the previously reported one [30], are simple, cost-effective, and non-hazardous. In situations where the measurement of a large number of samples is necessary, such as for monitoring the titers of medical personnel, the competitive ELISA, which is applicable to prevalent strains and has been confirmed to correlate with virus neutralization assay, will be widely applicable.

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Declaration of Competing Interest

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Appendix A. Supplementary data

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