Activation of SARS-CoV-2 neutralizing antibody is slower than elevation of spike-specific IgG, IgM, and nucleocapsid-specific IgG antibodies

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COVID-19 antibody testing has been developed to investigate humoral immune response in SARS-CoV-2 infection. To assess the serological dynamics and neutralizing potency following SARS-CoV-2 infection, we investigated the neutralizing (NT) antibody, anti-spike, and anti-nucleocapsid antibodies responses using a total of 168 samples obtained from 68 SARS-CoV-2 infected patients. Antibodies were measured using an authentic virus neutralization assay, the high-throughput laboratory measurements of the Abbott Alinity quantitative anti-spike receptor-binding domain IgG (S-IgG), semiquantitative anti-spike IgM (S-IgM), and anti-nucleocapsid IgG (N-IgG) assays. The quantitative measurement of S-IgG antibodies was well correlated with the neutralizing activity detected by the neutralization assay \( r = 0.8943, p < 0.0001 \). However, the kinetics of the SARS-CoV-2 NT antibody in severe cases were slower than that of anti-S and anti-N specific antibodies. These findings indicate a limitation of using the S-IgG antibody titer, detected by the chemiluminescent immunoassay, as a direct quantitative marker of neutralizing activity capacity. Antibody testing should be carefully interpreted when utilized as a marker for serological responses to facilitate diagnostic, therapeutic, and prophylactic interventions.

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a major public health concern. The reverse transcription polymerase chain reaction (RT-PCR) test is considered the gold standard for detecting the presence of viral RNA. However, the accuracy of RT-PCR relies heavily on sample collecting timing, type, storage, handling, and processing. RT-PCR products of SARS-CoV-2 nucleotides can be detected several days after onset1, with sensitivity declining after 1–2 weeks2. Conversely, the sensitivity of serological assays increases 2 weeks after symptom onset3, indicating combined RT-PCR and antibody testing can be complementary for laboratory diagnosis.

SARS-CoV-2 is composed of four structural proteins, spike (S), nucleocapsid (N), envelope (E), and membrane (M), and more than 20 nonstructural proteins. Of these, S and N proteins have been used as antibody assay targets3,4. N proteins are RNA-binding proteins consisting of nucleocapsids, which are highly immunogenic...
and expressed abundantly during infection. Therefore, antibodies that bind to N proteins can be indicators of exposure to the virus
due to the potential of N proteins in mediating SARS-CoV-2 entry into cells. The N protein includes the N-terminal domain (NTD) and receptor-binding domain (RBD), whereas the N2 protein promotes membrane fusion. The RBD is responsible for direct binding to angiotensin-converting enzyme 2 (ACE2), a host cell receptor responsible for mediating SARS-CoV-2 attachment. Because the RBD is predominantly targeted by the immune system, with 90% of the neutralizing activity of SARS-CoV-2 immune serum targeting RBD, anti-RBD antibodies have the potential to neutralize viral entry into cells and are crucial in the protective immune response to SARS-CoV-2 infection. Furthermore, NTD-specific antibodies have also been reported to neutralize SARS-CoV-2. These findings indicate that not only RBD, used as the current target of vaccines, but also NTD could be an attractive target for vaccine design.

With respect to the kinetics of neutralizing and anti-S protein antibodies in COVID-19 patients, the titers of both neutralizing and anti-S protein antibodies have been shown to be higher in symptomatic patients than in asymptomatic patients. While coordinated antibody responses with CD4+ T cell and CD8+ T cell are protective, uncoordinated responses fail to combat disease and show impaired immune responses to SARS-CoV-2. These findings indicate that humoral adaptive immune responses are stronger in critically ill patients, protective, uncoordinated responses fail to combat disease and show impaired immune responses to SARS-CoV-2. These findings indicate that not only RBD, used as the current target of vaccines, but also NTD could be an attractive target for vaccine design.

In this study, an authentic virus neutralization assay was used to investigate serological kinetics and neutralization potential after SARS-CoV-2 infection. The clinical performance of high-throughput, widely available laboratory measurements of the three serological assays for SARS-CoV-2 antibodies was then evaluated in comparison to neutralizing activity. The assays evaluated were S protein RBD-specific IgG antibody quantification reagents (S-IgG, anti-S Abbott SARS-CoV-2 IgG II Quant), whole S protein-specific IgM antibody semi-quantitative reagents (S-IgM, anti-S Abbott Alinity SARS-CoV-2 IgM), and semi-quantitative reagents for N protein-specific IgG antibody (N-IgG, anti-N Abbott Alinity SARS-CoV-2 IgG) and analyze how neutralizing antibody function evolves during infection and promote recovery.

**Results**

**Correlations between S-IgG, S-IgM, and N-IgG antibody levels and NT antibody activity.** To assess the potential utility of the tested serological assays, SARS-CoV-2 IgG II Quant assay (S-IgG), anti-S SARS-CoV-2 IgM assay (S-IgM), and anti-N SARS-CoV-2 IgG assay (N-IgG), we first validated their clinical specificity and linearity. The specificities of S-IgG, S-IgM, and N-IgG were evaluated using the samples collected before the COVID-19 pandemic. Clinical linearity was examined for the quantitative S-IgG assay using five COVID-19 patient samples with elevated S-IgG antibody values. We evaluated the linearity of the S-IgG assay using five samples with elevated S-IgG titer. The S-IgG assay showed excellent linearity up to the samples in which antibody value was 17,742.5 AU/ml (Supplementary Fig. S1A), just below the manufacturer-recommended clinical reportable range of 20,000 AU/ml.

The correlations of S-IgG, S-IgM, and N-IgG antibody levels with NT antibody activities detected by the authentic virus neutralizing assay were investigated using 141 samples, in which these antibodies’ titers were measured simultaneously. COVID-19 cases were divided into Group M, including mild and moderate cases, and Group S, including severe and critical cases, according to the WHO criteria. The quantitative S-IgG assay showed a strong correlation with the NT antibody titers (Group M, r = 0.8965, p < 0.0001; Group S, r = 0.8363, p < 0.0001) (Fig. 1). The semi-quantitative S-IgM and N-IgG also showed a positive correlation with NT activity (S-IgM, Group M, r = 0.8352, p < 0.0001; N-IgG, Group M, r = 0.7867, p < 0.0001; Group S, r = 0.7940, p < 0.0001). There were no clear differences in correlation between group S and group M.

As shown in Table 1, among the NT antibody negative samples (n = 39), 6 samples (15%) were positive for S-IgG, 4 (10%) were positive for S-IgM, and 3 (8%) were positive for N-IgG. Of the samples with NT antibody titers from 5 to 20 (n = 28), 4 samples (14%) were negative for S-IgG, 4 samples (14%) were negative for S-IgM, and 5 samples (18%) were negative for N-IgG. Samples with more than 20 titer of NT antibody were almost all positive for S-IgG, S-IgM, and N-IgG.

**Kinetics of NT antibody and S-IgG, S-IgM, and N-IgG antibodies after SARS-CoV-2 infection.** We then investigated the kinetics of the NT antibody and anti-S-IgG, anti-S-IgM, and anti-N-IgG antibodies using 168 longitudinally assessed samples from the 68 patients. Although all tested antibodies increased in the early phase of infection, neutralization titers of the patients in the severe group (Group S) achieved maximal responses later than the mild symptomatic cases (Group M). Figure 2 shows that maximal NT antibody activity was achieved 53 days after onset (1.48 logs titer) for Group M, while 69 days were required for Group S to develop a maximal response (1.79 logs titer). The speeds of reaching maximum levels of S-IgG and S-IgM antibodies were faster than that of NT antibodies (S-IgG: 3.87 logs at day 46 for Group M, 3.96 logs at day 45 for Group S; S-IgM: 0.96 logs at day 45 for Group M, 1.06 logs at day 49 for Group S). The maximal response of N-IgG antibody was achieved later for Group S (day 58, 0.75 logs) than for Group M (day 46, 0.90 logs).

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Longitudinal assessment in antibody titers. To examine changes in antibody levels over time, we plotted the titers of inpatients measured three or more times in a row (Fig. 3A). A total of 109 samples from 22 cases were collected up to 142 days after symptom onset to determine the change slopes of antibodies, as described in “Materials and methods”. Table 2 summarizes the clinical background characteristics.

One severely afflicted patient (Pt #13), who showed suppressed or delayed antibody responses, suffered from liver cancer with cytomegalovirus reactivation and could not be rescued (died after 37 days from onset). In this patient, S-IgG remained positive from day 24 with low titer near the positive threshold line (58.0–105.8 AU/
mum NT antibody titer is delayed in Group S, as shown in Fig. 2. No significant difference between Group M activated later than S-IgG, S-IgM, and N-IgG. This is consistent with the result that the timing to obtain maxi-
during T2–T3 for Group M, but not for Group S. These findings indicate that in severe cases, NT antibody is
S-IgG antibodies based on disease severity (Fig. 3B). Time point 1 (T1), T2, T3 and T4 were within the time
frames of 0–13 days, 14–27 days, 28–41 days, and 42–55 days after symptom onset, respectively. From T1 to T2,
S-IgG, S-IgM, and N-IgG antibodies increased more rapidly than in the period from T2 to T3 for both Group
M and Group S. We observed that NT antibodies increased with a significantly larger slope during T1–T2 than
during T2–T3 for Group M, but not for Group S. These findings indicate that in severe cases, NT antibody is
activated later than S-IgG, S-IgM, and N-IgG. This is consistent with the result that the timing to obtain maxi-
mum NT antibody titer is delayed in Group S, as shown in Fig. 2. No significant difference between Group M
and Group S was observed in all tested antibodies among the T1–T2, T2–T3, and T3–T4 slopes.

**Discussion**

Although NT antibodies are important for virus clearance and to achieve protection against SARS-CoV-2\(^ 22,23 \),
the kinetics of SARS-CoV-2 antibodies, including NT antibody upon infection, remain controversial.

Concordant with the previous report demonstrating a strong correlation between virus-neutralizing activity and
the level of anti-RBD antibodies that block SARS-CoV-2 entry to cells\(^ 24 \), we observed that levels of S-IgG
for RBD of S protein showed a stronger correlation with the NT antibody titers comparing to those of S-IgM and
N-IgG. However, this study showed that 15% of the NT antibody negative samples were positive for S-IgG
and that the development of NT antibody was slower in comparison to S IgG in severe COVID-19 cases.

These findings indicate that there may be a discrepancy between the antibody levels measured by serologic
testing and the neutralizing activity detected by authentic virus neutralization assay. It is known that through a
process named affinity maturation, the binding ability of virus-specific IgG antibody is relatively low in the early
stage of viral infection and increases with time\(^ 25,26 \), which is a consequence of B-cells somatic hypermutation. In
SARS-CoV-2 infection, anti-RBD antibodies also have been shown to increase in binding affinities and neutral-
izing potency over time\(^ 21,27 \). This process might be associated with lower NT antibody activity and a delayed
increase compared to S-IgG.

In contrast, Trinité et al. reported a rapid development of NT antibodies in ICU patients and no variations
in the kinetic activity between severe and non-severe participants\(^ 28 \). While the reason for the discrepant find-
ings is not clear, it could be attributed to the different patient conditions examined. Although we included only
inpatients in our longitudinal assessment study, Trinité et al. compared hospitalized severe patients to mild
symptomatic or asymptomatic patients that were not hospitalized\(^ 29 \). The methodological difference of NT anti-
body measurement might be another reason.

It is known that IgM is a potent activator of the classical complement pathway, and plays an important role
in the early stages of the immune response and disappears from peripheral blood earlier than IgG\(^ 30 \). However,
in this study, we observed that the seroconversion of anti-SARS-CoV-2 S-IgG and S-IgM antibodies occurred
simultaneously, which is concordant with previous reports\(^ 30,31,32 \). The seroconversion pattern of SARS-CoV-2
infection suggests the presence of cross-reactive immunity to previously induced general human coronavirus
encounters\(^ 33 \). Furthermore, it has been reported that existing T cell immunity to the common seasonal cold
coronaviruses (hCoV-229E, -NL63, -HKU1, and -OC43) can prime the response to SARS-CoV-2\(^ 34 \).

This study has several limitations. The first is the relatively small number of patients sampled, as well as gender
bias within this small sample. Of the 22 patients analyzed, only 4 were female. Because males are known to be
more prone to severe disease and higher immune responses due to several possible biological factors, such as
hormonal differences and sex-specific genes involved in viral recognition and immune response\(^ 35 \), it is possible
that gender bias could affect the results. Second, while we examined 100 specimens from the pre-COVID-19
period to determine assay specificity, the serological cross-reactivity in samples infected with various corona
and influenza viral infections should also be measured. Third, the samples used for determining the kinetics of
the specific antibodies, including NT antibody, were mainly obtained from hospitalized COVID-19 patients and
did not include asymptomatic cases. Larger cohorts with longer follow-up periods are required, and vaccination
further presents an opportunity to elucidate correlates of protection against SARS-CoV-2 infection.

| NT antibody (titers) | Positive/ negative | Sample number | S-IgG Positive (%) | S-IgG Negative (%) | S-IgM Positive (%) | S-IgM Negative (%) | N-IgG Positive (%) | N-IgG Negative (%) |
|---------------------|--------------------|----------------|------------------|-------------------|------------------|------------------|------------------|------------------|
| > 0, < 5            | Negative           | 39             | 6 (15)           | 33 (85)           | 4 (10)           | 35 (90)          | 3 (8)            | 36 (92)          |
| > 5, <10            | Positive           | 13             | 11 (85)          | 2 (15)            | 11 (85)          | 2 (15)           | 10 (77)          | 3 (23)           |
| > 10, < 20          | Positive           | 15             | 13 (87)          | 2 (13)            | 13 (87)          | 2 (13)           | 13 (87)          | 2 (13)           |
| > 20, < 40          | Positive           | 26             | 26 (100)         | 0 (0)             | 25 (96)          | 1 (4)            | 25 (96)          | 1 (4)            |
| > 40, < 80          | Positive           | 29             | 29 (100)         | 0 (0)             | 28 (97)          | 1 (3)            | 28 (97)          | 1 (3)            |
| > 80, < 160         | Positive           | 14             | 14 (100)         | 0 (0)             | 14 (100)         | 0 (0)            | 14 (100)         | 0 (0)            |
| > 160               | Positive           | 5              | 5 (100)          | 0 (0)             | 5 (100)          | 0 (0)            | 5 (100)          | 0 (0)            |

Table 1. Correlation between SARS-CoV-2 NT antibody activities and S-IgG, S-IgM, N-IgG antibody levels.
We observed that the quantitative measurement of S-IgG correlated well with the neutralizing activity detected by the neutralization assay. However, these S-IgG antibodies were not completely consistent because their antibody titers are not the same as the neutralization reaction activity.

In conclusion, this study demonstrated the limitation of using the S-IgG antibody titer detected by the chemiluminescent immunoassay as a direct quantitative marker of neutralization activity capacity. Careful interpretation should be given when using antibody test results as markers of serological responses to facilitate diagnosis, treatment, and prophylactic intervention.

Figure 2. Longitudinal change of antibodies against SARS-CoV-2. Scatterplot and regression lines indicate antibody response for longitudinal analysis: Group M (n = 95 from 52 patients) and Group S (n = 73 from 16 patients). (A) NT antibody, (B) S-IgG, (C) S-IgM, and (D) N-IgG. The 95% CIs are calculated by prediction ± 1.96 × standard error of prediction. The red lines indicate the points in which the fitted curves are at their maximum. These points are as follows: (A) NT antibody; Group M, day 53, log titer 1.48; Group S, day 69, 1.79, (B) S-IgG; Group M, day 46, 3.87; Group S, day 45, 3.96, (C) S-IgM; Group M, day 45, 0.96; Group S, day 49, 1.06, (D) N-IgG, Group M, day 46, 0.90, Group S, day 60, 0.75.
The diluted samples were mixed with the virus, whose titer in culture supernatant was $6.8 \times 10^7$ median tissue culture infectious dose (TCID50) per mL at 3 days post infection38, and then incubated at 37°C for 1 h.

Neutralization assay. The SARS-CoV-2 ancestral strain WK-521 (lineage A, GISAID ID: EPI_ISL_408667) was used for the authentic virus neutralization assay, which was performed at the National Institute of Infectious Diseases (NIID) with ethics approval by the medical research ethics committee of NIID for the use of human subjects (#1178). The virus neutralization assay was performed as described previously39. Briefly, serum samples were serially diluted: twofold serial dilutions starting at 1:5 dilution performed with high glucose Dulbecco’s Modified Eagle Medium supplemented with 2% Fetal Bovine Serum (Fujifilm Wako Pure Chemicals, Japan).

We first categorized SARS-CoV-2 infection patients as mild, moderate, severe, or critical according to the WHO criteria44. Mild COVID-19 was defined as respiratory symptoms without evidence of pneumonia or hypoxia, while moderate or severe infection was defined as the presence of clinical and radiological evidence of pneumonia. In moderate cases, SpO2 $\geq 94\%$ was observed on room air, while one of the following was required to identify the severe and critical cases: respiratory rate $>30$ breaths/min or SpO2 $<94\%$ on room air. Critical illness was defined as respiratory failure, septic shock, and/or multiple organ dysfunction (COVID-19 Clinical management: living guidance. [https://www.who.int/publications/i/item/clinical-management-of-covid-19]). We then stratified them into either Group M, which included mild and moderate cases, or Group S, which included severe and critical cases. Group M patients with a high-risk background were hospitalized and included in the long-term evaluation study. In this study complied with all relevant national regulations and institutional policies. It was conducted in accordance with the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board (IRB) at Juntendo University Hospital (IRB # 20-036). The need for informed consent from individual patients was waived by the Institutional Review Board (IRB) at Juntendo University Hospital because all samples were de-identified in line with the Declaration of Helsinki.

Serologic testing for SARS-CoV-2. Anti-S SARS-CoV-2 IgG II Quant assay (S-IgG), anti-S SARS-CoV-2 IgM assay (S-IgM), and anti-N SARS-CoV-2 IgG assay (N-IgG) were performed on the Abbott Alinity platform according to the manufacturer’s instructions. These are the chemiluminescent microsphere (CMIA) assays for quantitative assessment of anti S IgG, semi-quantitative assessment of anti S IgM antibodies, and semi-quantitative assessment of anti N IgG antibodies, respectively. In the S-IgG assay, the SARS-CoV-2 antigen-coated paramagnetic microparticles bind to the IgG antibodies that attach to the receptor binding domain (RBD) of the SARS-CoV-2 spike protein S1 subunit in human serum and plasma samples. The sequence used for the RBD was taken from the WH-Human 1 coronavirus (GenBank accession number MN908947)39,40. The S-IgM assay is designed to detect IgM antibody against the whole spike protein, including RBD (https://www.hsa.gov.sg/docs/default-source/hprg-mdh/psar-covid-19-puo-tests/ab03_abbott-alinity-i-sars-cov-2-igm.pdf)40. The resulting chemiluminescence in relative light units indicates the strength of the response, which reflects each specific antibody present. Results from the quantitative S-IgG assay are reported as arbitrary units (AU) per milliliter, and values equal to the cutoff of 50 AU/ml or greater were classified as positive41. Results from the semi-quantitative anti-S IgM and anti-N IgG assays are reported as index values, and the manufacturer’s suggested positive cutoff points of 1.0 and 1.40 were used, respectively40,42.
The clinical linearity of the quantitative anti-S SARS-CoV-2 IgG II Quant assay was evaluated using five patient samples with elevated S-IgG values. Samples were measured at 1:2 dilutions and compared to the theoretical values calculated with their undiluted sample results.

**Statistical analysis.** Data analysis was carried out using GraphPad Prism software (version 9.0.1; San Diego, CA, USA) and R software (version 4.1.0). Titers of antibodies were log-transformed before statistical analyses. Correlation analysis between antibody titer and neutralization test was performed using the Spearman correlation coefficient. For the longitudinal analysis, a nonlinear model was fitted for the kinetics of each SARS-CoV-2 antibody. The 95% confidence interval was calculated by performing bootstrap resampling. We conducted a decay analysis of each tested antibody between T1–T2, T2–T3, and T3–T4 for Group M and Group S.

The NT antibodies were log-transformed and the mean of the NT slopes for each patient at each time point (T1–T2, T2–T3, T3–T4) was plotted to visualize the increasing rate of the antibodies of each group. The rate of increase was examined by the Wilcoxon rank-sum test (Fig. 3B). P values of < 0.05 were considered statistically significant.

**Data availability**
All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Author contributions
T.N. and Y.T. designed the study. K.N.S., Y.B., K.R.S., G.I., T.Y., M.Y., K.S., T.K., M.T., and T.S. carried out data collection and analysis. M.T., T.A., S.N., S.T., Y.H. and Y.T. performed data analysis and wrote the manuscript. A.K. and F.J.P. contributed to writing and preparing the manuscript. M.H. contributed to sample collection. M.W., T.M., A.O., and K.T. discussed experiments and contributed to data interpretation; all authors critically reviewed and revised the manuscript for content and approved the manuscript for publication.

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Competing interests
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Additional information
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