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Neutralizing Antibody Responses to SARS-CoV-2 in Korean Patients Who Have Recovered from COVID-19

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Purpose: Neutralizing antibodies (NAbs) have been considered effective in preventing and treating viral infections. However, until now, the duration and clinical implications of antibody-mediated nature immunity in Koreans have remained unknown. Therefore, we examined NAbs levels and clinical characteristics in recovered coronavirus disease 2019 (COVID-19) patients.
Materials and Methods: Blood samples were collected from 143 adult patients who had been diagnosed with and had recovered from COVID-19 from February to March in 2020 at a tertiary-care university-affiliated hospital in Daegu, Korea. A plaque reduction neutralization test was conducted to analyze NAb titers. Individualized questionnaires were used to identify patient clinical information.
Results: The median number of days from symptom onset to the blood collection date was 109.0 (104.0; 115.0). The NAb titers ranged from 10 to 2560. The median NAb titer value was 40. Of the 143 patients, 68 (47.6%) patients had NAb titers ≥80, and 31 (21.7%) patients had NAb titers ≥160. The higher the age or disease severity, the higher the NAb titer. In univariate logistic regression, statistically significant predictors of high NAb titers (≥80) were age, myalgia, nausea or vomiting, dyspnea, and disease severity (p<0.05). Multivariable logistic regression showed that age ≥50 years (p=0.013) and moderate or higher disease severity (p<0.001) were factors associated with high NAb titers (≥80). None of the patients had reinfection of COVID-19.
Conclusion: All recovered patients were found to have NAbs regardless of the NAb titers maintained by natural immunity. Age and disease severity during COVID-19 infection were associated with high NAb titers.

Key Words: COVID-19, SARS-CoV-2, neutralizing antibodies, immunity, neutralization assay

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and development of the coronavirus disease 2019 (COVID-19) have presented a major healthcare challenge of global dimensions. Neutralizing antibodies (NAbs) are important for viral clearance and are considered key to recovery and protection against viral diseases. Depending on the isotype (IgM, IgA, IgG, etc.), NAbs have different roles in viral clearance and disease prevention.[1,2] One previous study showed that the presence of NAbs from prior infection was significantly associated with protection against reinfection,[3] and NAbs levels have been used as a standard with which to evaluate the efficacy of vaccines.[4] Passive antibody therapy has been used as a potential therapy for infectious viral diseases, where the efficacy of such therapy was found to be associated with the concentration of NAbs in the plasma of recovered donors,[5] and studies have reported that convalescent plasma therapy could be a potentially effective treatment option for

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patients hospitalized with COVID-19.[1,6,7] Therefore, identifying the characteristics, including titers, of NAbs in Koreans who have recovered from COVID-19 could help conduct proper convalescent plasma therapy.

The United States Food and Drug Administration guidelines have issued emergency use authorization for COVID-19 convalescent plasma to treat hospitalized patients with COVID-19. NAbs titers in convalescent plasma should be at least 160, although titer levels of 80 are acceptable in the absence of a better match.[8] Currently, there are only a few studies available on protective immunity to SARS-CoV-2 and the factors associated with NAbs level. One study demonstrated that severe COVID-19 is associated with higher antibody production and neutralization titers and that the development of neutralizing humoral immunity against SARS-CoV-2 appears to increase survival.[9] In the aforementioned study, of 113 patients, only 18 did not require hospitalization. Further studies are needed to determine how the immune responses in relation to NAbs operate in mild cases, which account for most COVID-19 infections. In addition to severity, it is also necessary to identify other factors related to NAbs titers. Therefore, we first measured SARS-CoV-2-specific NAb titers in the plasma of 143 patients who had recovered from COVID-19 and determined the antibody-positive persistency rate. We then investigated associations between NAb titers and clinical characteristics.

MATERIALS AND METHODS

Study design and population

Daegu city was the first area where a severe COVID-19 outbreak was identified in the Republic of Korea from February to March 2020.[10] We screened recovered COVID-19 patients using the patient data registry from the Daegu Center for Infectious Diseases Control and Prevention. To confirm the possibility of participation in the study and to arrange blood sampling hospital visits, cellular phone text messages were sent to recovered COVID-19 patients in Daegu. Individuals who agreed to participate in NAb-tracking study were recruited from the Kyungpook National University Hospital located in Daegu city. We included patients diagnosed with COVID-19 between February 19 to March 28, 2020. Patients aged <18 years or those who were unable to make a voluntary decision to participate in the research, such as patients with mental illness, were excluded. From June 3 to June 23, 2020, blood samples were collected from 143 polymerase chain reaction (PCR)-confirmed COVID-19-recovered patients. On the day of hospital visitation, a survey of clinical characteristics was conducted using an individualized questionnaire that assessed basic demographics, clinical symptoms at COVID-19 diagnosis, persisted symptoms until the survey date, and admission information. Confirmation of the original diagnosis of SARS-CoV-2 and epidemiological information were provided by the Daegu Center for Infectious Diseases Control and Prevention. Clinical data, including the first symptom onset date, disease severity, and underlying diseases from the survey, were reevaluated and matched with the Daegu Center for Infectious Diseases Control and Prevention data registry.

The characteristics of symptoms at COVID-19 diagnosis included fever, sore throat, anosmia, ageusia, myalgia, cough, sputum, rhinorrhea, diarrhea, nausea or vomiting, dyspnea, and headache.

Sequelae category included cough, sputum, throat discomfort, dyspnea, chest discomfort, ageusia, anosmia, fatigue, dizziness, obsessive thinking, insomnia, hallucination, anxiety, paresthesia, cognitive dysfunction, abnormal directional sensibility, palpitation, arrhythmia, diarrhea, febrile sense, chilly sense, swollen toes, and muscle ache. Underlying diseases included allergic rhinitis, diabetes mellitus, hypertension, chronic kidney disease, liver disease, hematologic malignancy, solid tumor, cerebrovascular accident, chronic obstructive pulmonary disease, and heart disease (heart failure, arrhythmia, valvular heart disease, and coronary heart disease).

This study was performed according to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines.

Definitions

SARS-CoV-2 infection was confirmed by real-time reverse transcription-PCR assay performed using nasopharyngeal swabs or other upper respiratory tract specimens.[11] COVID-19 clinical severity classification was defined on the basis of clinical information as follows[10]: 1) asymptomatic, no symptoms or discomfort throughout the disease period, with body temperature <37.5°C; 2) mild, the presence of any symptoms with or without fever (≥37.5°C), but not manifesting pneumonia; 3) moderate, pneumonia diagnosed by a clinician, but not requiring oxygenation other than room air; 4) severe, pneumonia diagnosed by a clinician and requiring oxygenation therapy (nasal prong, facial mask, or high-flow oxygen therapy); and 5) critical, pneumonia diagnosed by a clinician and requiring mechanical ventilation therapy or extracorporeal membrane oxygenation or death. High NAb titer was defined as NAb titers higher than the median value. Sequelae in this study were defined as newly identified symptoms after COVID-19 diagnosis that persisted until the survey date. Reinfection with COVID-19 was defined as clinical recurrence of symptoms compatible with COVID-19 accompanied by positive PCR (Ct<35) at more than 90 days after the onset of the primary infection supported by close contact exposure or outbreak settings and no evidence of another cause of infection.[12]

Materials and assays

Approximately 10 mL of whole blood was collected in tubes containing acid citrate dextrose. The blood samples were separated into plasma and peripheral blood mononuclear cells by centrifugation within 12 h of collection. The plasma samples

were immediately frozen at -70°C. All samples were transported in a frozen storage form. The plaque reduction neutralization test (PRNT) was conducted at the Department of Microbiology of Korea University in Seoul, where a bio-safety level three facility is available. The neutralization activity of plasma from COVID-19-recovered patients was analyzed by PRNT. The SARS-CoV-2 of S clade (BetaCoV/Korea/KCDC03/2020, NCCP 43326) was used for neutralization activity analysis. The neutralization assay was performed in accordance with the following steps: In the PRNT measurement, the antibody sample was diluted with phosphate-buffered saline at 1:10 and further serially diluted two-fold in stages. The diluted antibody samples (100 μL each) were mixed with the same volume of virus (100 PFU in 100 μL) and reacted for 1 h at 37°C. After 1 h of reaction, the whole reaction mixture was used to inoculate Vero cells for the subsequent plaque assay. Virus-inoculated Vero cell plates (NEST Scientific, #703003; SPL Life Sciences, Pochen, Korea) were overlaid with agar and incubated at 37°C, 5% CO_2 for 3 days. The plates were then stained with crystal violet (GeorgiaChem, #548-6-29; Georgia Chemicals Inc, Norcross, GA, USA) to visualize the plaques formed by replicating non-neutralized infectious virus. PRNT50 was determined as the maximum antibody dilution fold value that reduced the number of plaques by >50%, compared with the control that was untreated with antibodies.

Statistical analysis

Continuous variables are represented as medians (interquartile range, IQR), and categorical variables are presented as numbers (percentage, %). Categorical variables were compared using Fisher's exact test or chi-square test with yates' continuity correction, and noncategorical variables were analyzed using Student's t-test or Mann-Whitney U-test. ANOVA or the Kruskal-Wallis test was performed in the difference test for age and disease severity index. To determine the factors associated with high NAb titer (≥80), univariate logistic regression analysis was performed. Among these predictors, multiple logistic regression by the stepwise method was used to determine factors contributing significantly to the prediction model. All tests were considered statistically significant at $p<0.05$. Statistical analyses were performed using R statistics v.4.0 (The R Foundation; https://www.r-project.org, Vienna, Austria).

Ethics statement

This study was approved by the Institutional Review Board of Kyungpook National University Hospital (approval no.: 2020-05-004). All participants signed an informed consent approved by the IRB and received financial compensation.

RESULTS

Description of the study population

Of 143 patients, 68 (47.6%) patients were male, and 75 (52.4%) patients were female. Regarding the age distribution, 34 (23.8%) patients were aged 20–29 years, 24 (16.8%) patients were aged 30–39 years, 25 (17.5%) patients were aged 40–49 years, 27 (18.9%) patients were aged 50–59 years, and 33 (23.1%) patients were aged 60–69 years. The median number of days from symptom onset to blood collection was 109.0 (104.0; 115.0). All samples collected from the 143 patients demonstrated neutralizing activity against SARS-CoV-2. The median value of NAb titers was 40. Fig. 1 shows the distribution of NAb titers in COVID-19-recovered patients. Seventy-five (52.4%) patients had NAb titers <80, and 68 (47.6%) patients had NAb titers ≥80. Regarding disease severity, there were 8 (5.6%) patients asymptomatic patients, 90 (62.9%) patients had mild disease, 29 (20.3%) patients had moderate disease, 12 (8.4%) patients had severe disease, and 4 (2.8%) patients were critical. In total, 135 (94.4%) patients were identified with clinical symptoms at COVID-19 diagnosis. Five (3.5%) patients had been admitted to the intensive care unit (ICU), whereas 16 (11.2%) patients had received oxygen treatment during the disease progress. Underlying diseases were confirmed in 68 (47.6%) patients. Sequelae were identified in 78 (54.5%) patients. None of the patients was identified to have symptomatic reinfection of COVID-19 (Table 1).

Disease severity and characteristics of NAb titers according to age

The higher the age, the higher the NAb titers (Fig. 2), and the proportion of patients with high titers of NAb increased with increasing age ($p<0.001$) (Supplementary Fig. 1, only online). 7 (20.5%) patients aged 20–29 years, 10 (41.7%) patients aged 30–39 years, 9 (36.0%) patients aged 40–49 years, 20 (74.0%) patients aged 50–59 years, and 22 (66.6%) patients aged 60–69 years were found to have NAb median titers ≥80 ($p<0.001$) (Supplementary Fig. 2, only online). It is notable that none of the

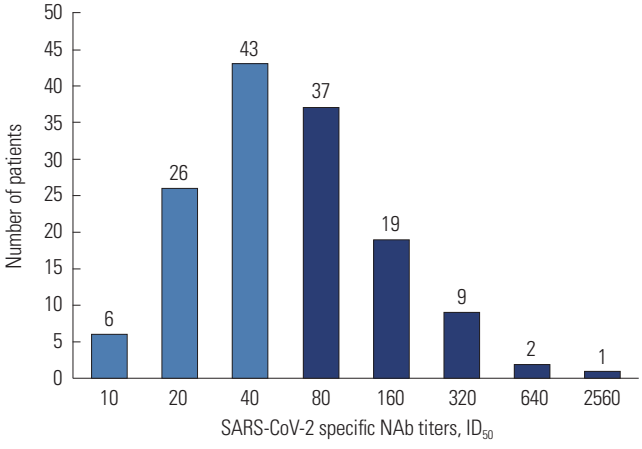

Fig. 1. Distribution of SARS-CoV-2-specific NAb titers (ID_{50}) in COVID-19-recovered patients. The number of patients is shown in the middle of each bar graph. The median NAb value was 40. Blue indicates NAb <80, and dark-blue indicates NAb ≥80. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; NAb, neutralizing antibody; ID_{50}, 50% inhibitory dose; COVID-19, coronavirus disease 2019.

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patients with NAb titers <10 was identified in the age group of 60–69 years. Moreover, 1 (3.0%) patient aged 60–69 years was found to have high NAb titers of 2560 [Supplementary Fig. 1 (only online) and Table 1].

The higher the age, the higher the proportion of patients with a higher level of disease severity during COVID-19 hospitalization with statistical significance (p<0.001) (Supplementary Fig. 3, only online). Twelve (44.4%) patients aged 40–49 years and 21 (63.6%) patients aged 60–69 years were moderate or higher severity cases. More than half of patients over 50 years were

Table 1. Clinical Characteristics of the Study Population according to Age Group

	20–29 yr (n=34)	30–39 yr (n=24)	40–49 yr (n=25)	50–59 yr (n=27)	60–69 yr (n=33)	Total (n=143)	*p* value
Sex							0.849
Female	19 (55.9)	12 (50.0)	13 (52.0)	16 (59.3)	15 (45.5)	75 (52.4)	
Male	15 (44.1)	12 (50.0)	12 (48.0)	11 (40.7)	18 (54.5)	68 (47.6)	
Height, cm	169.5 [158.0; 173.0]	169.5 [161.0; 177.0]	166.0 [158.0; 174.0]	164.0 [158.5; 171.0]	162.0 [158.0; 169.0]	166.0 [158.0; 172.0]	0.177
Weight, kg	64.0 [54.0; 70.0]	70.0 [59.0; 81.0]	68.0 [55.0; 75.0]	66.0 [58.0; 70.5]	64.0 [57.0; 70.0]	65.0 [56.0; 72.5]	0.201
Severity category							<0.001
Asymptomatic	3 (8.8)	3 (12.5)	2 (8.0)	0 (0.0)	0 (0.0)	8 (5.6)	
Mild	30 (88.2)	14 (58.3)	19 (76.0)	15 (55.6)	12 (36.4)	90 (62.9)	
Moderate	0 (0.0)	6 (25.0)	3 (12.0)	10 (37.0)	10 (30.3)	29 (20.3)	
Severe	1 (2.9)	1 (4.2)	1 (4.0)	1 (3.7)	8 (24.2)	12 (8.4)	
Critical	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.7)	3 (9.1)	4 (2.8)	
NAb titer							<0.001
10	1 (2.9)	2 (8.3)	1 (4.0)	2 (7.4)	0 (0.0)	6 (4.2)	
20	14 (41.2)	7 (29.2)	2 (8.0)	1 (3.7)	2 (6.1)	26 (18.2)	
40	12 (35.3)	5 (20.8)	13 (52.0)	4 (14.8)	9 (27.3)	43 (30.1)	
80	6 (17.6)	9 (37.5)	6 (24.0)	9 (33.3)	7 (21.2)	37 (25.9)	
160	1 (2.9)	1 (4.2)	1 (4.0)	5 (18.5)	11 (33.3)	19 (13.3)	
320	0 (0.0)	0 (0.0)	2 (8.0)	5 (18.5)	2 (6.1)	9 (6.3)	
640	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.7)	1 (3.0)	2 (1.4)	
2560	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.0)	1 (0.7)	
NAb titer							<0.001
<40	15 (44.1)	9 (37.5)	3 (12.0)	3 (11.1)	2 (6.1)	32 (22.4)	
≥40	19 (55.9)	15 (62.5)	22 (88.0)	24 (88.9)	31 (93.9)	111 (77.6)	
Duration*	107.0 [102.0; 113.0]	107.5 [101.5; 116.5]	112.0 [104.0; 116.0]	106.0 [103.0; 109.0]	115.0 [107.0; 117.0]	109.0 [104.0; 115.0]	0.012
Diagnosed as COVID-19 after symptom onset							0.088
Yes	23 (67.6)	21 (87.5)	18 (72.0)	23 (85.2)	30 (90.9)	115 (80.4)	
No	11 (32.4)	3 (12.5)	7 (28.0)	4 (14.8)	3 (9.1)	28 (19.6)	
Symptoms during COVID-19							0.155
Yes	31 (91.2)	21 (87.5)	23 (92.0)	27 (100.0)	33 (100.0)	135 (94.4)	
No	3 (8.8)	3 (12.5)	2 (8.0)	0 (0.0)	0 (0.0)	8 (5.6)	
Sequelae of COVID-19							<0.001
Yes	9 (26.5)	11 (45.8)	13 (52.0)	18 (66.7)	27 (81.8)	78 (54.5)	
No	25 (73.5)	13 (54.2)	12 (48.0)	9 (33.3)	6 (18.2)	65 (45.5)	
ICU admission							0.036
Yes	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.7)	4 (12.1)	5 (3.5)	
No	34 (100.0)	24 (100.0)	25 (100.0)	26 (96.3)	29 (87.9)	138 (96.5)	
Oxygen treatment							<0.001
Yes	1 (2.9)	1 (4.2)	1 (4.0)	2 (7.4)	11 (33.3)	16 (11.2)	
No	33 (97.1)	23 (95.8)	24 (96.0)	25 (92.6)	22 (66.7)	127 (88.8)	
Presence of underlying diseases							<0.001
Yes	7 (20.6)	9 (37.5)	9 (36.0)	18 (66.7)	25 (75.8)	68 (47.6)	
No	27 (79.4)	15 (62.5)	16 (64.0)	9 (33.3)	8 (24.2)	75 (52.4)	

IQR, interquartile range (range from lower to upper quartile); NAb, neutralizing antibody; ICU, intensive care unit; COVID-19, coronavirus disease 2019.
Data are presented as n (%) or median [IQR].
*Duration from symptom onset or diagnosis to the blood sample collection.

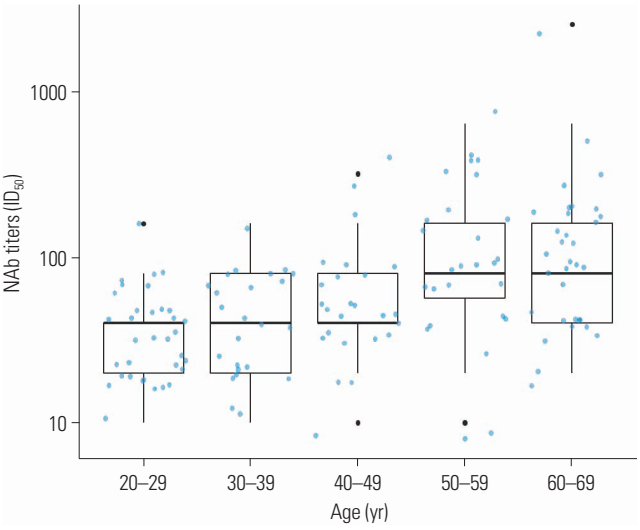

Fig. 2. NAb titers (ID_{50}) with categorized age groups in COVID-19-recovered patients. The higher the age, the higher the NAb titers were. NAb, neutralizing antibody; ID_{50}, 50% inhibitory dose; COVID-19, coronavirus disease 2019.

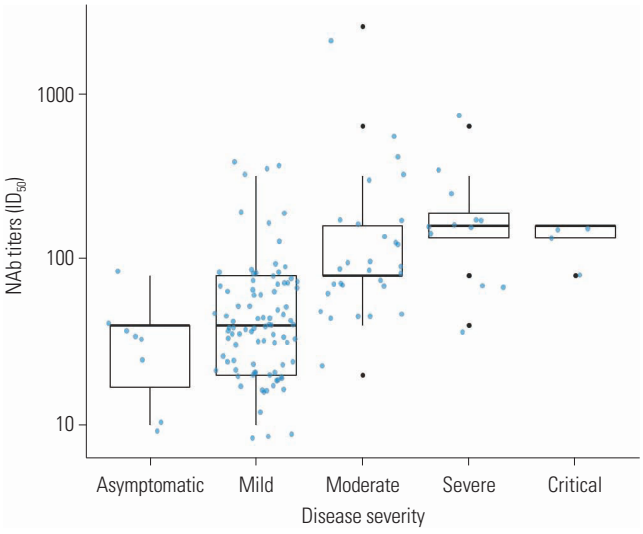

Fig. 3. SARS-CoV-2 specific NAb titers (ID_{50}) according to disease severity. The higher the disease severity, the higher the NAb titers were. NAb, neutralizing antibody; ID_{50}, 50% inhibitory dose; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

moderate or higher severity cases. Alternatively, 1 (2.9%) patient in the age group of 20–29 years, 1 (4.2%) patient in the age group of 30–39 years, and 1 (4.0%) patient in the age group of 40–49 years were severe disease cases. In this study, none of the patients younger than 50 years had critical disease severity, whereas 1 (3.7%) patient aged 50–59 years and 3 (9.1%) patients aged 60–69 years were critical cases. Three (8.8%) patients aged 20–29 years, 3 (12.5%) patients aged 30–39 years, and 2 (8.0%) patients aged 40–49 years were asymptomatic cases, whereas none of the patients aged 50 years or older were asymptomatic (Table 1). In terms of sequelae, the higher the age, the higher the proportion of patients who suffered from sequelae, even after a median of 109 days after COVID-19 (p<0.001). Moreover, the proportion of patients with ICU hospitalization (p=0.036) and oxygen treatment (p<0.001) were higher in older patients (Table 1).

Clinical characteristics according to disease severity

The higher the degree of disease severity, the higher the NAb titers of the patient (Fig. 3). The proportion of patients with high titers of NAb increased with increasing disease severity (p<0.001) (Supplementary Fig. 4, only online). Five (62.5%) patients in the asymptomatic group, 62 (68.9%) patients in the mild group, 28 (96.6%) patients in the moderate group, 12 (100%) patients in the severe group, and 4 (100%) patients in the critical group showed a median NAb titer value of 40 or more [Supplementary Fig. 4 (only online) and Table 2]. The duration from symptom onset or diagnosis to blood sample collection and duration from COVID-19 diagnosis to the end of quarantine showed no statistically significant association with the five disease severity groups. However, the higher the severity, the higher the proportion of patients complaining of sequelae after a median of 109 days from COVID-19 infection (p=0.004) (Table 2).

Factors associated with high NAb titers (≥80)

Seventy-five (52.4%) patients had NAb titers <80, and 68 (47.6%) patients had titers ≥80. The incidence of symptoms of myalgia (p=0.048), nausea or vomiting (p=0.039), and dyspnea (p<0.001) were significantly higher in the patient groups with NAb titers ≥80 (Supplementary Table 1, only online). Furthermore, the number of patients treated with oxygen was statistically significant at NAb titers ≥80 (p<0.001). There were no statistical differences in sex and underlying diseases between the two titers groups. To determine factors associated with NAb titers ≥80, univariate and multivariate logistic regression analyses were performed. In univariate logistic regression, patients aged ≥50 years were significantly more common in the NAb titers ≥80 group than in the NAb titers <80 group [61.8% (42/68) vs. 24.0% (18/75), p<0.001]. Patients with moderate or higher disease severity were significantly more frequent in the NAb titers ≥80 group than in the NAb titers <80 group [55.9% (38/68) vs. 9.3% (7/75)]. In multivariate analysis, age ≥50 years (p=0.031) and moderate or higher disease severity (p=0.006) were identified as independent factors associated with NAb titers ≥80 (Table 3).

DISCUSSION

In this observational study, NAb titers in 143 patients who recovered from COVID-19 were investigated using PRNT. The titers of SARS-CoV-2-specific NAbs varied substantially, and 47.6% of them had NAb titers ≥80. The higher the age or disease severity, the higher the NAb titers were. Factors associated with NAb titers ≥80 were age ≥50 years and moderate or higher disease severity.

Virus-specific NAbs are critical determinants for viral clearance.[13-15] The spike protein receptor-binding domain is the most

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immune-dominant neutralizing epitope eliciting virus neutralization, and NAb titers may vary depending on amino acid substitutions of viral spike protein variations.[16] This study discovered that NAb titers ≥80 were associated with age (≥50 years) or disease severity (moderate or higher). This result can be referenced in selecting plasma therapy donors with a high probability for higher levels of NAb. However, high levels of antibodies with low neutralization potency could potentially increase

Table 2. Clinical Characteristics of the Study Population according to Disease Severity

	Asymptomatic (n=8)	Mild (n=82)	Moderate (n=37)	Severe (n=12)	Critical (n=4)	Total (n=143)	*p* value
Sex							0.183
Female	4 (50.0)	46 (51.1)	20 (69.0)	4 (33.3)	1 (25.0)	75 (52.4)	
Male	4 (50.0)	44 (48.9)	9 (31.0)	8 (66.7)	3 (75.0)	68 (47.6)	
Height, cm	169.0 [161.0; 173.0]	167.0 [158.0; 173.0]	162.0 [158.0; 170.0]	167.0 [159.5; 172.0]	161.0 [158.0; 169.5]	166.0 [158.0; 172.0]	0.787
Weight, kg	69.0 [57.5; 79.0]	65.0 [56.0; 73.0]	64.0 [57.0; 69.0]	69.5 [65.0; 74.5]	62.5 [58.0; 68.0]	65.0 [56.0; 72.5]	0.440
Age, yr							<0.001
20–29	3 (37.5)	30 (33.3)	0 (0.0)	1 (8.3)	0 (0.0)	34 (23.8)	
30–39	3 (37.5)	14 (15.6)	6 (20.7)	1 (8.3)	0 (0.0)	24 (16.8)	
40–49	2 (25.0)	19 (21.1)	3 (10.3)	1 (8.3)	0 (0.0)	25 (17.5)	
50–59	0 (0.0)	15 (16.7)	10 (34.5)	1 (8.3)	1 (25.0)	27 (18.9)	
60–69	0 (0.0)	12 (13.3)	10 (34.5)	8 (66.7)	3 (75.0)	33 (23.1)	
NAb titer							<0.001
10	2 (25.0)	4 (4.4)	0 (0.0)	0 (0.0)	0 (0.0)	6 (4.2)	
20	1 (12.5)	24 (26.7)	1 (3.4)	0 (0.0)	0 (0.0)	26 (18.2)	
40	4 (50.0)	33 (36.7)	5 (17.2)	1 (8.3)	0 (0.0)	43 (30.1)	
80	1 (12.5)	21 (23.3)	12 (41.4)	2 (16.7)	1 (25.0)	37 (25.9)	
160	0 (0.0)	4 (4.4)	6 (20.7)	6 (50.0)	3 (75.0)	19 (13.3)	
320	0 (0.0)	4 (4.4)	3 (10.3)	2 (16.7)	0 (0.0)	9 (6.3)	
640	0 (0.0)	0 (0.0)	1 (3.4)	1 (8.3)	0 (0.0)	2 (1.4)	
2560	0 (0.0)	0 (0.0)	1 (3.4)	0 (0.0)	0 (0.0)	1 (0.7)	
NAb titer							0.004
<40	3 (37.5)	28 (31.1)	1 (3.4)	0 (0.0)	0 (0.0)	32 (22.4)	
≥40	5 (62.5)	62 (68.9)	28 (96.6)	12 (100.0)	4 (100.0)	111 (77.6)	
Duration*	105.5 [102.0; 110.5]	108.0 [104.0; 115.0]	108.0 [103.0; 114.0]	114.0 [109.5; 117.0]	108.0 [99.0; 114.0]	109.0 [104.0; 115.0]	0.347
Diagnosed as COVID-19 after symptom onset							<0.001
Yes	0 (0.0)	74 (82.2)	27 (93.1)	10 (83.3)	4 (100.0)	115 (80.4)	
No	8 (100.0)	16 (17.8)	2 (6.9)	2 (16.7)	0 (0.0)	28 (19.6)	
Symptoms during COVID-19							<0.001
Yes	0 (0.0)	90 (100.0)	29 (100.0)	12 (100.0)	4 (100.0)	135 (94.4)	
No	8 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	8 (5.6)	
Sequelae of COVID-19							0.004
Yes	0 (0.0)	47 (52.2)	19 (65.5)	8 (66.7)	4 (100.0)	78 (54.5)	
No	8 (100.0)	43 (47.8)	10 (34.5)	4 (33.3)	0 (0.0)	65 (45.5)	
ICU admission							<0.001
Yes	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)	4 (100.0)	5 (3.5)	
No	8 (100.0)	90 (100.0)	29 (100.0)	11 (91.7)	0 (0.0)	138 (96.5)	
Oxygen treatment							<0.001
Yes	0 (0.0)	0 (0.0)	0 (0.0)	12 (100.0)	4 (100.0)	16 (11.2)	
No	8 (100.0)	90 (100.0)	29 (100.0)	0 (0.0)	0 (0.0)	127 (88.8)	
Presence of underlying diseases							0.015
Yes	2 (25.0)	36 (40.0)	18 (62.1)	10 (83.3)	2 (50.0)	68 (47.6)	
No	6 (75.0)	54 (60.0)	11 (37.9)	2 (16.7)	2 (50.0)	75 (52.4)	

IQR, interquartile range (range from lower to upper quartile); NAb, neutralizing antibody; ICU, intensive care unit; COVID-19, coronavirus disease 2019.
Data are presented as n (%) or median [IQR].
*Duration from symptom onset or diagnosis to the blood sample collection.

Table 3. Logistic Regression Analyses of Factors Associated with NAb Titers ≥80

Factors	Univariate analysis		Multivariable analysis	
	OR (95% CI)	*p* value	OR (95% CI)	*p* value
Female	1.46 (0.75–2.83)	0.264	-	-
Age 50 years or above	5.12 (2.53–10.73)	<0.001	2.81 (1.25–6.38)*	0.013
Myalgia	2.14 (1.07–4.35)	0.033	-	-
Diarrhea	1.88 (0.90–4.03)	0.096	-	-
Nausea or vomiting	3.80 (1.25–14.19)	0.027	-	-
Dyspnea	9.68 (3.46–34.61)	<0.001	-	-
Severity moderate or higher	12.30 (5.20–32.99)	<0.001	8.56 (3.45–23.69)*	<0.001

OR, odds ratio; CI, confidence interval.
*Results of the stepwise method.

clinical severity of COVID-19.[9] Therefore, using convalescent plasma as a treatment strategy requires attention because severely ill patients can have low neutralization potency. Also, further studies are needed to determine whether sera from COVID-19-convalescent patients can reduce the severity of the disease in high-risk populations.

The relationship between disease severity and NAb titers has been widely investigated. In patients who have recovered from asymptomatic COVID-19, antibody-negative rates were found to be higher in the convalescent phase than in patients who had symptoms [12/37 (40.0%) vs. 4/37 (12.9%)].[17] In the present study, 1 (12.5%) patient out of eight asymptomatic patients and 67 (49.6%) patients of 135 symptomatic patients were identified to have NAb titers of 80 or higher. A study conducted on ICU and non-ICU patients demonstrated that the ICU patients had an accelerated and augmented NAb response, compared with non-ICU patients, that was associated with disease severity.[18] According to another cohort study conducted in China on 59 patients, on the 20th day of symptom onset, NAb responses were found to be correlated with disease severity. However, only four patients with mild symptoms were enrolled.[19] Another study of NAbs in relation to the severity of disease after 2 months from symptom onset or laboratory diagnosis reported that NAb titers were correlated with the disease's severity.[20] Our study, including patients with various degrees of disease severity, ranging from asymptomatic to ICU hospitalized, showed a relation between high NAb titers and increased clinical disease severity, even after a median of 109 days after symptom onset.

Wu, et al.[21] reported that 10 (6.0%) of 175 patients showed NAb titers below a detectable level with neutralization assay (<40), even 2 weeks after hospital discharge. However, interestingly, a previous study showed that some asymptomatic seropositive individuals could generate NAb titers >1000 at an average of 39 days after the onset of symptoms, and highly potent neutralizing monoclonal antibodies have been isolated from asymptomatic patients.[22] Recent research conducted in London found that some individuals maintain NAbs titers >1000 at >60 days post-onset of symptoms, whereas others have NAbs titers approaching baseline within 94 days.[23] There is a need for an attempt to determine the factors associated with the persistency of high NAb titers. Studies seeking to identify factors associated with high NAb titers have been conducted on eight ICU patients and 42 non-ICU patients in China. Multivariate analysis revealed that oxygen requirement and fever were the only factors associated with a higher NAb response.[18] One study conducted on 126 patients demonstrated that male sex, advancing age, and hospitalization were associated with increased antibody responses across serological assays.[24] However, in our study, while sex was not associated with high NAb titers, age and disease severity were factors associated with NAb titers ≥80. One study conducted in Shanghai, China demonstrated that older patients had significantly higher titers of NAbs than younger patients at the time of discharge.[25] Another study reported that older patients with COVID-19 are at higher risk of developing severe and critical disease than younger adults.[26] Our study also revealed similar results: older age (≥50) was associated with NAb titers ≥80 in COVID-19-recovered patients at a median of 109 days after the date of symptom onset. A possible explanation for this finding is that older patients with COVID-19 infection tend to have more severe diseases than younger patients, and enhanced inflammatory responses associated with increased disease severity could drive higher B-cell recruitment and consequently more antibody production. Therefore, the magnitude of antibody responses may also likely be correlated with disease severity. This hypothesis suggests that patients with more severe diseases are more protected against reinfection: those with asymptomatic or mild disease could be more vulnerable to waning immunity over time because the initial immune response was not as strong as that in patients with more severe disease.[20]

One retrospective cohort study showed that prior infection in patients with COVID-19 was highly protective against reinfection (≥90 days after initial testing) and that symptomatic disease results in protection rates of 81.8% and 84.5%, respectively. However, the NAb detection was not implemented in this study.[27] Therefore, till date, the protective level of NAb titers in humans has not yet been identified. In an animal study in ferrets, direct-contact transmission was observed only from reinfected ferrets with low NAb titers (<20).[28] In our study, no pa-

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tient had symptomatic reinfection with COVID-19, even in patients with NAb titers <20. However, we should also consider environmental conditions that may result in the possibility of a decrease in social activities among recovered COVID-19 patients than in non-infected individuals due to sequelae or a more precautious attitude, thereby preventing COVID-19 reinfection, or national restrictions of mandatory mask wearing at public facilities. It would be important to follow up with patients to evaluate protective NAb titer levels, which may have a significant implication on vaccination strategies in recovered COVID-19 patients around the world. Simultaneously, understanding NAb responses is clinically important, especially in relation to the use of convalescent plasma or hyperimmune globulin therapy.[18]

Our study has several limitations. First, since NAb titers were measured at one cross-sectional point, and NAb development kinetics could not be detected from the acute COVID-19 period. Therefore, we could not identify if NAb titers were lower in milder patients or if NAb titers had waned. However, we showed that the NAb responses depend on age and disease severity. Second, there were only 12 and four patients in the severe and critical disease groups, respectively. Thus, the small number of patients could limit the interpretation of our study results. However, this study included patients aged 60–69 years with mild or severe cases concurrently and demonstrated results from the largest number of patients in each specified age group. Third, all patients included in this study were adults, and it is necessary to investigate NAb responses in children. Fourth, clinical information, such as laboratory findings during hospitalization or specific treatments, including steroids, that may affect NAb titers was excluded. In regards to NAb measurements applied in this study, since the S clade was targeted, further NAb titer research on the other clade or variant SARS-CoV-2 strain and on utilization according to NAb isotype will be needed. Also, analysis of the qualitative characteristics of antibodies and potency index are required.

In conclusion, after a median of 109 days of COVID-19, recovered patients were found to have varying NAb titer levels, and none of the patients experienced reinfection of COVID-19. To prepare convalescent serum antibody treatment and to establish appropriate targeted immunization strategies, continuous tracking of changes in NAbs is needed.

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