Dynamics of Neutralizing Antibodies and Binding Antibodies to Domains of SARS-CoV-2 Spike Protein in COVID-19 Survivors

Supinya Phakaratsakul

Mahidol University Faculty of Medicine Siriraj Hospital

Suwimon Manopwisedjaroen

Mahidol University Faculty of Science

Chompunuch Boonarkart

Mahidol University Faculty of Medicine Siriraj Hospital

Pawinee Kupatawintu

Thai Red Cross Society

Dootchai Chaiwanichsiri

Thai Red Cross Society

Thaneeya Roytrakul

Mahidol University Faculty of Medicine Siriraj Hospital

Prasert Auewarakul

Mahidol University Faculty of Medicine Siriraj Hospital

Arunee Thitithanyanont (✉ arunee.thi@mahidol.ac.th)

Mahidol University Faculty of Science <https://orcid.org/0000-0002-9756-4763>

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Abstract

Neutralizing antibody level is used to predict immune protection against SARS-CoV-2 infection. Spike protein of SARS-CoV-2 is a major target for virus-neutralizing antibody. A number of neutralizing epitopes were mapped on receptor binding domain (RBD) and N-terminal domain (NTD) of S1 subunit of the spike. Anti-SARS-CoV-2 antibody usually decreases over time after recovery. Level of neutralizing antibody and binding antibody to several domains from COVID-19 recovered patients were observed longitudinally in this study. Sequentially collected serum samples from 35 patients demonstrated both similar and different trends of neutralizing antibodies versus binding antibodies to each domain. Twenty-three individuals showed similarly decreasing pattern of neutralizing titer, binding antibodies to RBD, NTD, fusion protein (S2) and nucleocapsid (NP). Interestingly, eight individuals had stably high neutralizing titer (≥ 320) for 3-12 months, while their binding antibodies to RBD, NTD and NP rapidly decreased. Moreover, their binding antibodies to S2 were stable over time similar to the persistence of neutralizing antibody levels. The longer-lasting antibody to S2 suggested an anamnestic response to cross-reactive epitopes from previous infections with other related coronaviruses. These data indicate a difference in kinetics and longevity of antibodies to various domains and epitopes of the SARS-CoV 2 proteins. A better understanding in this difference may help improve vaccine design to induce long-lasting immunity to COVID-19.

Introduction

The humoral immune response to SARS-CoV-2 has been widely studied since neutralizing antibody is a hope for anti-viral protection and treatment. A number of neutralizing epitopes were characterized. Potent neutralizing antibodies mainly target epitopes on spike glycoprotein (S) of SARS-CoV-2 (1–5). Each monomer of trimeric S-protein consists of S1 and S2 subunits. Receptor binding domain (RBD) located in the S1 subunit specifically interacts with human angiotensin converting enzyme 2 (ACE2) to mediate cell entry. RBD is considered the main target for efficient neutralization (4–7). N-terminal domain (NTD) is also located on the S1 subunit and interacts with sialic acids or coreceptors. The neutralizing antibodies targeting NTD is usually less prevalent than those targeting RBD. S2 subunit mediates fusion of the viral and cell membrane. It is highly conserved among corona viruses and S2-targeting neutralizing antibodies may inhibit formation of six-helix bundle structure and consequently block membrane fusion (8, 10). Nucleocapsid (NP), located inside the virion, is responsible for RNA packaging and virus replication. It might be important in modulating antiviral immunity and inhibiting interferon production (11).

Neutralizing efficacy relies on binding position and affinity of antibodies (10). SARS-CoV-2 variants with mutations in RBD and NTD exhibited partially resistance to neutralizing antibodies generated by natural infection or vaccination (12). Delta variant (B.1.617.2), containing diverse mutations in the RBD and NTD, was resistant to neutralization by some anti-NTD and anti-RBD monoclonal antibodies and convalescent sera from COVID-19 patients (13).

The duration of protective immunity to SARS-CoV-2 plays a pivotal role in rates of reinfection and vaccine breakthrough and hence the overall trajectory of the pandemic. Neutralizing antibody dropped rapidly in the first 2-3 months after infection and slower thereafter (14, 15). While NP and S-specific IgG titers declined, the potent neutralizing activity sustained for up to 12 months after natural infection in about 20% of patients (16, 17). Further investigations in the long-lasting antibody of COVID-19 patients might provide important insights leading to novel approaches in vaccine design.

Here, we characterized longitudinal trend of binding antibodies to several domains of SARS-CoV-2 that may be responsible for the neutralizing activities in COVID-19 recovered patients. Micro-neutralization of SARS-CoV-2 and ELISA analysis of binding antibody were tested in 307 convalescent sera which were sequentially collected from 35 patients from 2 to 12 months after recovery. Binding levels of the sera to SARS-CoV-2 RBD, NTD, S2 and NP antigens were determined and compared with neutralizing titers.

Materials And Methods

Serum samples

The study included 307 sera from 35 COVID-19 recovered patients during February 2020 to April 2021 provided by Thai Red Cross Society. The blood collection was part of the activity to prepare convalescent hyperimmunized plasma for COVID-19 treatment. For negative control, sera collected prior SARS-CoV-2 pandemic (in 2010) were used. The study was approved by the ethic committees of the Thai Red Cross and the Faculty of Medicine Siriraj Hospital.

Live-virus micro-neutralization assay

Live-virus micro-neutralization of SARS-CoV-2 using a cell-based indirect ELISA detecting NP of virus was performed in a certified Biosafety Level 3 (BSL-3) facility at the Department of Microbiology, Faculty of Science, Mahidol University. The experimental protocols were performed following standard protocols approved by the Biosafety committee of Mahidol University. SARS-CoV-2 viruses (SARS-CoV-2/01/human/Jan2020/Thailand) representing the original Wuhan strain isolated from a confirmed COVID-19 patient at Bamrasnaradura Infectious Diseases Institute, Nonthaburi, Thailand and the B.1.617.2 delta variant (SARS-CoV-2/human/THA/VTM1_P2/2021) were used for in vitro experiment. In brief, heat-inactivated serum was two-fold serially diluted in culture medium starting at 1:10 and 60 μ l and mixed with 60 μ l (100 TCID₅₀) SARS-CoV-2 for 1 hour. The mixture was then added to Vero-E6 cells and incubated for 1 hour. The cells were washed and further incubated in medium for 48 hours. Cytopathic effect was observed under microscope and cells were fixed with 1:1 cold methanol/acetone for 20 min. The fixed cells were stained using SARS-CoV-2 NP monoclonal antibody (Sino Biological) and a secondary peroxidase-labeled goat anti-rabbit IgG (Dako). Signal was developed using TMB substrate (KPL) and color was halted by adding 1N HCl. The plate was read by an ELISA reader at wavelength of 450 nm and 620 nm. The average OD_{450/620} is determined for quadruplicate wells of 100 TCID₅₀ and negative control wells (CC), and a neutralizing endpoint is determined by using a 50% specific signal calculation. The endpoint titer was expressed as the reciprocal of the highest dilution of serum with average OD 450/620 value (duplicate wells) less than X. $X = [(average\ O.D.\ of\ 100TCID_{50}) - (average\ O.D.\ of\ CC)] / 2 + (average\ O.D.\ of\ CC)$.

ELISA analysis of antibody binding to SARS-CoV-2 spike and nucleocapsid antigens

SARS-CoV-2 RBD, NTD, S2 or NP protein (Sino Biological) at 0.5 µg/ml was coated on a MaxiSorp Nunc-immuno 96-well plate overnight at 4°C. Wells were blocked with blocking buffer (5% BSA and 0.1% Tween-20 in PBS) for 1 h at room temperature, followed by incubation with 1:2000 diluted sera in blocking buffer for 1 h at 37°C. Wells were washed four times with washing buffer (0.1% Tween-20 in PBS). A 1:5000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody (Thermo) was added for 1 h at 37°C. Wells were washed four times with washing buffer and developed using KPL TMB microwell peroxidase substrate (Seracare) for 10 min at room temperature. The reaction was stopped by adding 1M H₂SO₄. Absorbance was read at 450 and 630 nm using microplate reader.

Results

We followed 35 individuals with confirmed history of COVID-19 who were willing to donate blood regularly and had neutralizing antibody titers over 160 at the first blood collection. Demographic data, onset duration before blood donation, symptoms and severity of individuals were showed in Table 1.

Table 1

Demographic data, onset duration before blood donation, symptoms and severity of 35 donors with confirmed history of COVID-19 who had neutralizing antibodies to SARS-CoV-2 over 160 at the first blood collection.

No.	Donor ID	NT titer	Sex	Age	Onset duration before blood donation (day)	Symptom (1=Yes, 0=No)						Pneumonia	ICU	Treatment
						Fever	Cough	Sore throat	Running nose	Hard breathing	Others			
1	LAB63V00020	1280	M	55	58	1	1	0	0	0	0	No	No	Receive medication according to symptom
2	LAB63V00022	160	M	26	56	1	1	1	1	1	0	No	No	Receive medication according to symptom
3	LAB63V00026	2560	M	38	58	1	1	0	0	0	0	No	No	Receive medication according to symptom
4	LAB63V00029	2560	M	43	56	1	1	0	0	0	0	Yes	No	Receive antiviral drug
5	CCP63000001	640	M	30	41	0	0	0	0	0	0	No	No	Receive medication according to symptom
6	CCP63000003	1280	M	42	22	0	0	0	1	0	0	No	No	Receive medication according to symptom
7	CCP63000005	160	M	42	54	1	1	0	0	0	0	No	No	Receive medication according to symptom
8	CCP63000026	640	M	46	62	1	0	1	0	0	0	No	No	Receive antiviral drug
9	CCP63000027	640	M	51	61	0	0	0	0	0	0	No	No	Receive medication according to symptom
10	CCP63000038	160	M	26	49	1	1	0	1	0	0	No	No	Receive antiviral drug
11	CCP63000046	640	F	38	53	0	0	0	0	0	0	No	No	Receive antiviral drug
12	CCP63000049	320	M	37	60	1	1	0	0	0	0	Yes	No	Receive medication according to symptom
13	CCP63000050	640	M	38	53	1	1	0	0	1	Muscle pain	Yes	Yes	Receive oxygen therapy
14	CCP63000053	160	M	29	67	1	1	0	0	0	0	No	No	Receive medication according to symptom

No.	Donor ID	NT titer	Sex	Age	Onset duration before blood donation (day)	Symptom (1=Yes, 0=No)						Pneumonia	ICU	Treatment
						Fever	Cough	Sore throat	Running nose	Hard breathing	Others			
15	CCP63000055	640	M	46	60	0	0	0	0	1	0	No	No	Receive medication according to symptom
16	CCP63000056	1280	M	48	60	1	0	0	0	0	0	No	Yes	Receive antiviral drug
17	CCP63000057	320	M	50	60	0	0	0	0	0	0	No	Yes	Receive antiviral drug
18	CCP63000061	640	M	33	55	0	1	0	0	0	0	No	No	Receive medication according to symptom
19	CCP63000064	1280	M	48	49	1	0	0	0	0	0	No	No	Receive antiviral drug
20	CCP63000070	1280	F	39	58	1	1	0	0	1	0	No	No	Receive medication according to symptom
21	CCP63000073	1280	M	37	65	1	1	1	1	0	Allergic rash	No	No	Does not receive medication
22	CCP63000077	320	M	34	53	1	0	0	0	1	0	No	Yes	Receive antiviral drug
23	CCP63000095	160	M	27	52	1	1	0	0	0	0	No	No	Receive medication according to symptom
24	CCP63000096	160	M	58	47	1	0	0	0	0	0	No	No	Receive medication according to symptom
25	CCP63000100	160	F	38	71	1	0	0	0	0	0	No	No	Receive medication according to symptom
26	CCP63000113	160	M	39	72	1	1	1	0	0	Anorexia	No	Yes	Receive medication according to symptom
27	CCP63000114	160	M	37	64	1	0	0	0	0	Diarrhea	No	No	Receive medication according to symptom
28	CCP63000118	640	M	48	70	0	1	1	0	1	Diarrhea	No	No	Receive medication according to symptom
29	CCP63000138	640	F	33	75	1	1	1	0	1	0	Yes	No	Receive medication according to symptom

No.	Donor ID	NT titer	Sex	Age	Onset duration before blood donation (day)	Symptom (1=Yes, 0=No)						Pneumonia	ICU	Treatment
						Fever	Cough	Sore throat	Running nose	Hard breathing	Others			
30	CCP63000153	320	M	34	67	1	1	0	0	1	0	No	Yes	Receive medication according to symptom
31	CCP63000158	160	M	29	34	1	1	0	0	1	0	Yes	No	Receive medication according to symptom
32	CCP63000166	320	M	29	29	1	0	0	0	0	0	No	Yes	Receive medication according to symptom
33	CCP63000189	2560	M	24	85	1	1	1	0	1	Diarrhea	No	Yes	Receive medication according to symptom
34	CCP63000194	640	F	53	68	1	1	0	0	0	0	No	No	Receive medication according to symptom
35	CCP63000209	320	F	35	69	1	0	1	0	0	0	Yes	Yes	Receive oxygen therapy

Blood samples were collected every 2-3 weeks and tested for neutralizing antibody titers, and the sequential blood collection was terminated once the neutralizing antibody titer declined to lower than 160. Figures 1 showed patterns of neutralizing antibody titers and binding antibody levels of RBD, NTD, S2 and NP of the individuals. Sera from donors in figure 1a exhibited similar trend between neutralizing titer and binding level of RBD, NTD, S2 and NP which mostly decreased over time. The neutralizing antibody titers decreased to lower than 160 within 3 months after the first blood collection in 9 donors. Another 3 donors ID CCP63000038, CCP63000114 and LAB63V00029 had neutralizing antibody titers below 160 after 13, 33 and 23 weeks, respectively. The neutralizing activities of sera from these donors probably belonged to all of these epitopes at the periods of collection.

There were 10 donors with high neutralizing antibody titers in figure 1b but they were lost to follow-up after 2 months leaving 13 donors with sequential antibody titer data over 3-12 months. These 13 donors showed persistently high neutralizing titer over than 3 months. Interestingly, donor ID. CCP63000073 and CCP63000189 still had high neutralizing titer at 640 and 1280, respectively, at 1 year after the first collection. As RBD is generally a major target of SARS-CoV-2-neutralizing antibodies, similar trend between neutralizing titers and binding levels of RBD were observed in donor ID LAB63V00020, LAB63V00022, CCP63000005, CCP63000026, CCP63000046, CCP63000077, CCP63000095, CCP63000118, CCP63000194 and CCP63000209. However, donor ID CCP63000050, CCP63000055, CCP63000073, CCP63000096, CCP63000113, CCP63000138, CCP63000166 and CCP63000189 had persistently high neutralizing titer (≥ 320), but their binding levels to RBD rapidly decreased. This decline of the RBD binding antibody levels in these subjects was faster than the decline of antibody to the other domains. As time passed, the dramatically decreasing of binding antibody to RBD continued while neutralizing titers were still maintained. This suggested that other epitopes were also crucial.

Binding antibody level of NTD mostly decreased over time except sera of donor ID CCP63000077, CCP63000113, CCP63000118, CCP63000138 and CCP63000194 which corresponded to the persistently high neutralizing titer. However, donor ID CCP63000027 and LAB63V00020 always had low binding level of NTD since the first collection. Their neutralizing antibodies may target NTD much lesser than other epitopes. Although binding antibody level of NP usually dropped over time, donor ID CCP63000114, CCP63000189 and CCP63000194 demonstrated similar trends between to NP and S2, which also corresponded to their neutralizing titers.

Surprisingly, binding antibody specific to S2 was always stable or slowly decreased in most of donors in this study. Donor ID CCP63000055, CCP63000077 and CCP63000189 clearly demonstrated long lasting and high neutralizing titer ≥ 640 with the high and constant level of binding antibody to S2 for 35, 43 and 47 weeks, respectively. We also tested these sera for neutralizing titers against a delta variant isolate and found that these sera with high neutralizing titers and high S2-binding antibody did not show significantly higher cross neutralization to the delta variant than sera with lower S2-binding antibody (Table2). This suggested that the persistent neutralizing activity mainly targeted variable S1 epitopes despite the low binding antibody to the NTD and RBD domains.

Overall, the antibody response to each epitope can be differently maintained over time after infection. The persistent neutralizing titer despite the low level of binding antibody to RBD was probably from the specific binding antibodies to non-RBD epitopes such as S2, NP and even quaternary epitopes. For natural infection, RBD seems to be mainly responsible in earlier periods and other epitopes may play roles for neutralizing activities in later periods.

Table 2
NT titer and binding antibody level of RBD, S2, NP and NTD epitope

Donor ID	Collection time of serum (week)	NT titer		Optical density of ELISA binding			
		Wuhan	Delta	RBD	S2	NP	NTD
Donors with persistently high NT and S2-binding antibodies but declining RBD-binding antibody.							
CCP63000055	1	640	80	1.474	2.068	1.491	1.863
CCP63000441	31	320	40	0.463	1.995	0.461	0.770
CCP63000138	1	640	20	1.526	2.414	1.732	1.421
CCP63000395	17	320	20	0.638	2.180	1.115	1.123
CCP63000166	1	320	<10	1.985	1.676	1.639	1.293
CCP63000446	30	320	<10	0.424	1.871	0.981	0.797
Donors with persistently high NT and RBD-binding antibodies.							
CCP63000142	3	640	80	1.910	1.241	1.305	1.195
CCP63000404	20	320	<10	1.162	1.201	1.213	0.659
CCP63000018	2	640	320	2.219	1.455	1.565	1.593
CCP63000276	10	640	80	1.989	1.216	1.296	1.042
LAB63V00026	1	2560	80	2.009	1.170	1.809	2.017
CCP63000330	12	640	20	1.428	1.363	0.829	1.249
Donors with rapidly decreasing NT and RBD-binding antibodies.							
CCP63000009	2	640	20	2.012	1.195	1.944	0.975
CCP63000027	1	640	80	0.995	0.840	1.308	0.259
LAB63V00029	1	2560	80	2.339	1.101	2.031	1.403

Discussion

In general, immune protection against COVID-19 can be implied by the level of neutralizing antibody against the virus. Among all of the SARS-CoV-2 proteins, RBD of S1 is the major target for the development of potent neutralizing antibody (18). Escape variants carrying mutations and deletions in RBD and NTD could reduce sensitivity to antibody neutralization. For instant, highly concerned mutations in RBD including E484K and K417N/T in B.1.351 (Beta) and P.1 (Gamma) variants were reported that they partially impair neutralization generated by previous infection or vaccination (12, 19). Additionally, neutralizing antibody titer of most of the convalescent sera correlated to level of specific binding antibody to RBD. Thus many anti-RBD assays were developed to use as a predictor of the neutralization capability (20–22).

Nevertheless, SARS-CoV-2 spike protein contains other important antigenic sites. Monoclonal antibodies isolated from COVID-19 patients were potent and diverse, and there were monoclonal antibodies targeting RBD, NTD and quaternary epitope on top of the spike (23). Besides RBD region, NTD and S2 might contribute to the neutralization as well (9, 10). S1-targeting monoclonal antibody 4A8 isolated from COVID-19 recovered patients demonstrated that it did not block the interaction between ACE2 and S protein, but exhibits high levels of neutralization against both authentic and pseudotyped SARS-CoV-2. This monoclonal antibody was proved to bind NTD (24). Antibody targeting S2 may be also important for the disease outcome. It was shown that IgG and IgA antibody repertoire less recognized RBD and fusion peptide in the S2 domain in severe and death COVID-19 cases (25).

For natural infection, anti-SARS-CoV-2 antibody usually decreases over time after disease recovery. Neutralizing antibody titers reached their peak at 10-15 days after disease onset and subsequently declined (9). Our results showed the long lasting high neutralizing antibody for a year in some donors. The neutralizing antibodies seem to mainly target RBD at the early period after recovery for at least 3 months. Binding antibodies to NTD, S2 and NP also corresponded to the neutralizing titers. On the other hands, some donors who still had high neutralizing titer for the longer period at 3-12 months showed a discrepancy between the stable neutralizing titers and the declining levels of RBD, NTD and NP-binding antibodies. Only binding antibody to S2 persisted over time. Our result showing persistence of S2-binding antibody is in agreement with a previous report showing stable S2 antibody over time (26).

Antibodies targeting epitopes on S2 have not got much attention in their neutralizing capability. However, a study showed that IgM at days 21 and 42 preferentially recognized epitopes on S2, followed by NTD and limited binding to epitopes in RBD (25). Antibodies to spike reached a peak by 5-8 weeks and then declined, while those to S2 of seasonal beta coronaviruses continued to rise and correlated significantly with neutralizing antibodies (27). In fact, S2 is more conserved among coronaviruses than S1. The high and stable level of anti-S2 is probably from anamnestic boost of cross-reactive neutralizing antibodies against S2 which preexist in populations since previous exposures to human coronaviruses (28). This cross-reactive antibody epitope in S2 was also suggested to reduce the severity of COVID-19 (3).

Conclusion

Antibody responses to SARS-CoV-2 recognize several epitopes of the virus. Antibodies to different epitopes could have different rate of decline over time, and their contribution to neutralizing activity could change. Although RBD is such a dominant epitope that can instantly elicit potent neutralizing antibodies, anti-RBD might not be long lasting. The mechanism governing the differential longevity of different antibodies is not well understood and further studies into the longevity of specific antibody may help us improve vaccine design to induce long-lasting immunity to COVID-19.

Declarations

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Compliance with ethical standards

Conflict of interest

The authors declare no conflicts of interest.

Research involving human participants and/or animals

Collection of human blood samples was approved by the Ethics Committee of the Faculty of Medicine Siriraj Hospital (Siriraj Institutional Review Board), which is an AAHRPP (Association for the Accreditation of Human Research Protection Programs)-accredited ethical committee. The study was performed under protocol COA No. Si 483/2020 "Measurement of neutralizing antibody in convalescent plasma donors for COVID-19 treatment", and was in full compliance with the Declaration of Helsinki and the Belmont Report. All the human subjects provided written informed consent.

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Figures

Fig. 1a

Fig. 1b

Figure 1

Comparison of neutralizing antibody titers and binding antibody levels of RBD, NTD, S2 and NP of sequentially collected sera from 35 donors. The sera from 12 donors showed neutralizing titer decreased over time to lower than 160 (a). The sera from 23 donors showed persistently high neutralizing antibody titers ≥ 160 over time (b).