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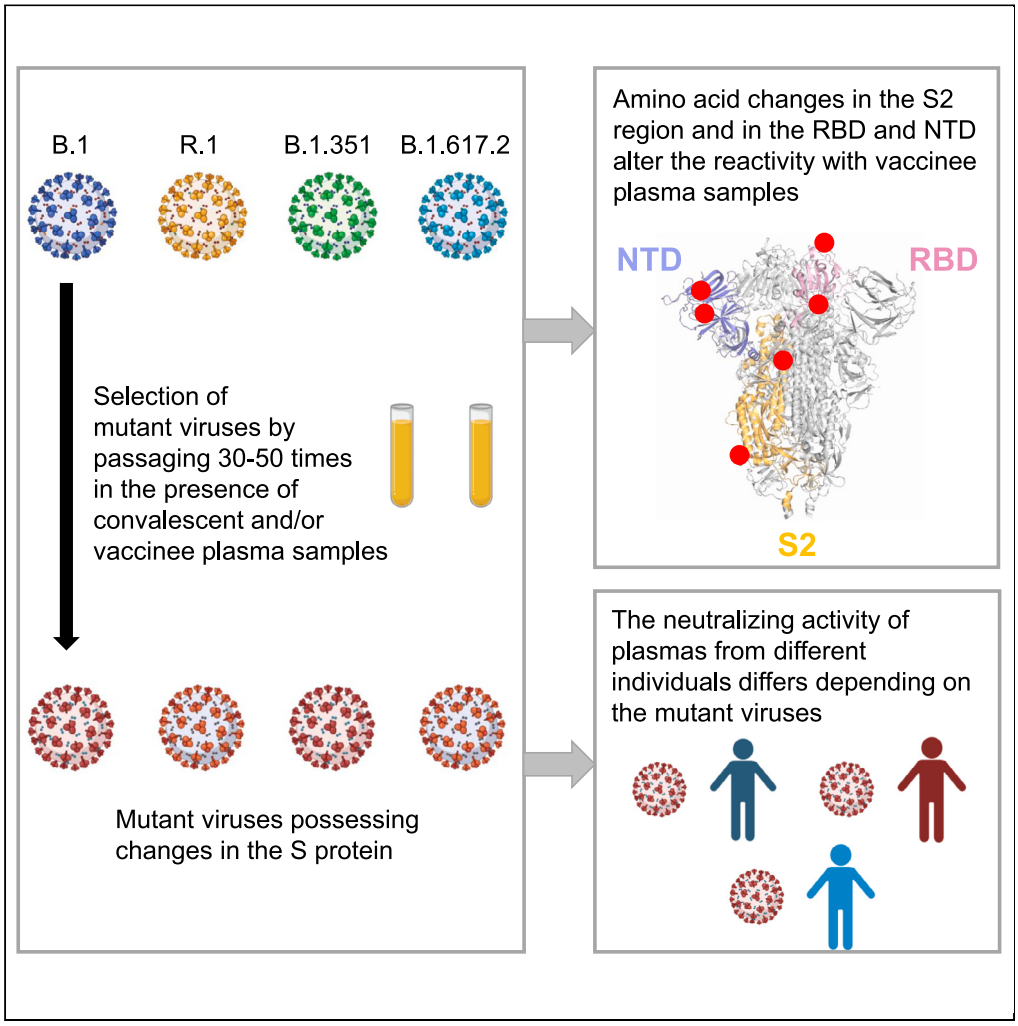

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Highlights

Amino acid changes in the S2 region alter the reactivity with vaccinee plasma samples

The neutralizing potency of convalescent or vaccinee plasmas varies among individuals

Genetic and immunological backgrounds affect the neutralizing epitope recognition

Yamamoto et al., iScience 26, 107208
July 21, 2023
https://doi.org/10.1016/j.isci.2023.107208

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Differences among epitopes recognized by neutralizing antibodies induced by SARS-CoV-2 infection or COVID-19 vaccination

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SUMMARY

SARS-CoV-2 has gradually acquired amino acid substitutions in its S protein that reduce the potency of neutralizing antibodies, leading to decreased vaccine efficacy. Here, we attempted to obtain mutant viruses by passaging SARS-CoV-2 in the presence of plasma samples from convalescent patients or vaccinees to determine which amino acid substitutions affect the antigenicity of SARS-CoV-2. Several amino acid substitutions in the S2 region, as well as the N-terminal domain (NTD) and receptor-binding domain (RBD), affected the neutralization potency of plasma samples collected from vaccinees, indicating that amino acid substitutions in the S2 region as well as those in the NTD and RBD affect neutralization by vaccine-induced antibodies. Furthermore, the neutralizing potency of vaccinee plasma samples against mutant viruses we obtained or circulating viruses differed among individuals. These findings suggest that genetic backgrounds of vaccinees influence the recognition of neutralizing epitopes.

INTRODUCTION

The pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has continued worldwide. To control the pandemic, vaccines and antivirals have been developed and have reduced the severity and mortality of SARS-CoV-2 infection, especially among the elderly.[1–3] Variants of concern (VOCs) such as alpha (lineage B.1.1.7), beta (B.1.351), gamma (P.1), delta (B.1.617.2), and omicron (B.1.1.529)[4] have emerged. Such VOCs possess many amino acid substitutions in the spike (S) protein of SARS-CoV-2 that reduce the potency of the neutralizing antibodies that are induced by vaccination and SARS-CoV-2 infection.[5,6] Although the vaccines reduce the severity of COVID-19,[7] novel VOCs that escape from the neutralizing antibodies induced by vaccination and infection may continue to emerge.

The S protein, especially the S1 subunit, is the major target of neutralizing antibodies induced by vaccination and infection.[8] The S1 subunit contains the receptor-binding domain (RBD) and the N-terminal domain (NTD). Several amino acid substitutions in the RBD have been shown to affect the potency of neutralizing antibodies.[9–18] The K417N substitution eliminates the neutralization activity of some anti-RBD antibodies.[9,14] The L452R substitution in the receptor-binding motif (RBM) of RBD confers partial escape from convalescent sera with a 2.0- to 6.7-fold reduction in neutralization titers.[12,13] The E484K substitution in the RBM sharply decreases the neutralization titers of convalescent plasma.[9,14,15] The delta variant possesses L452R, the omicron variants possess K417N, and isolates of lineage R.1 and the beta/gamma variants possess E484K.[4] Similarly, amino acid substitutions or deletions in the NTD are associated with a partial escape from neutralizing antibodies.[19–23] An amino acid substitution at position 18 (L18F), which is located in the supersite of the NTD, is associated with escape from anti-NTD neutralizing antibodies isolated from convalescent patients.[19] Deletion of H69/V70 in the N2 loop of the NTD or deletion of Y144 in the N3 loop of the NTD also affects the neutralizing activity of anti-NTD antibodies.[19,23] Alpha variants possess both deletions and omicron variants possess the H69/V70 deletion, whereas beta and gamma variants possess the L18F substitution.[4] Although these amino acid substitutions contribute to escape from

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neutralizing antibodies, we do not fully understand why these specific substitutions were selected. Furthermore, it is not clear whether amino acid substitutions that occur in regions other than the NTD and RBD, such as the S2 subunit, contribute to the escape from neutralizing antibodies.

One to two nucleotide mutations per month have been identified in the SARS-CoV-2 genome during the pandemic.[24–27] Many amino acid substitutions have been introduced into the S protein of the VOCs. Such hypermutations can occur during persistent infection of immunocompromised patients[28] with uncontrolled AIDS or cancer, or in solid organ transplant recipients.[29–32] In these patients, persistent viral replication coupled with an inadequate immune response has the potential to facilitate the emergence of hypermutated viruses with antigenic changes.[28]

To understand the evolutionary mechanism of mutations with antigenic change, we selected mutant viruses by passaging SARS-CoV-2 in the presence of plasma samples from convalescent patients or vaccinees and examined the antigenicity of these mutant viruses by using plasma samples collected from another cohort.

RESULTS

Selection of mutant viruses partially escaped from neutralizing antibodies in individuals who were infected with SARS-CoV-2 and/or received two doses of Pfizer mRNA vaccine

To examine whether amino acid substitutions found in VOCs were selected by immune pressure in COVID-19 patients or vaccinees, we passaged authentic SARS-CoV-2 B.1, R.1, B.1.351 (beta variant), and B.1.617.2 (delta variant) in the presence of vaccinee or convalescent plasma. First, we chose plasma samples that had neutralizing antibodies against SARS-CoV-2 (lineage B.1) or a high ELISA titer against the RBD of SARS-CoV-2 (lineage A) from plasma samples obtained from COVID-19 patients at about three months after symptom onset (Table S1), individuals who received two doses of Pfizer mRNA vaccine (Table S2), and individuals who were infected with SARS-CoV-2 and then received two doses of Pfizer mRNA vaccine (Table S3). Four convalescent plasma samples (HICo-002, HICo-004, HICo-011, and KYCo-001), two vaccinee plasma samples (HP[H]-008 and HP[H]-024), and two plasma samples from infected-then-vaccinated individuals (SUCo-85 and SUCo-86) showed relatively high neutralization or ELISA titers in each group. Therefore, we used these eight plasma samples for the selection of mutant viruses. Before the selection, we measured the neutralization titers of the eight plasma samples against B.1 and R.1 viruses or B.1.351 (beta variant) and B.1.617.2 (delta variant) viruses (Table 1). Next, The B.1 and R.1 viruses were passaged 50 or 40 times in triplicate in the presence of convalescent plasma (HICo-002, HICo-004, HICo-011, and KYCo-001), whereas the B.1.351 (beta variant) and B.1.617.2 (delta variant) viruses were passaged 30 times in triplicate in the presence of vaccinee and/or convalescent plasma (HP[H]-008, HP[H]-024, SUCo-85, and SUCo-86). After 30–50 passages, the nucleotide sequences of the S gene were analyzed to identify amino acid substitutions that occurred during passaging. For the mutants in which several amino acid substitutions were detected, the nucleotide sequences of the S gene were determined every tenth passage to determine the order in which the substitutions were introduced. The sequence analyses revealed that the amino acid substitutions occurred at various regions and differed depending on the starting viruses and plasma samples used (Table 1).

Neutralization titers of vaccinee plasma samples against the emerged mutant viruses

To clarify whether the amino acid changes identified during passages in the presence of the plasma samples affect the antigenicity of the S protein, we performed a neutralization assay using 10 plasma samples (HP[H]-010, HP[H]-191, HP[H]-197, HP[H]-200, HP[H]-217, HP[H]-218, HP[H]-228, HP[H]-250, HP[H]-279, and SU[H]-08) with high neutralization and ELISA titers selected from another mRNA-vaccinated cohort (Table S4). Among the B.1-derived mutant viruses, neutralization titers against the A67V, W64G plus A67V, L141F, L517F, A1174T, and A1174T plus K921E mutant viruses were significantly lower than those against wild-type virus (Figure 1A and Table S5). Neutralization titers against the A67V or A1174T mutant virus were similar to those against the W64G plus A67V or K921T plus A1174T mutant virus. These results indicate that the A67V, L141F, L517F, and A1174T substitutions affect the neutralization potency of vaccinated individuals. The A67V and L141F substitutions are located in the NTD of the S protein, the L517F substitution is located in the RBD of the S protein, and the A1174T substitution is located in heptad repeat 2 (HR2) (Figures 1A and 2A). Among the R.1-derived mutant viruses, neutralization titers against the F140del and K921Q mutant viruses were significantly lower than those against wild-type virus (Figure 1B and Table S6). These results indicate that the F140del and K921Q mutations affect the neutralization potency of vaccinated individuals. The F140del mutation is in the NTD and the K921Q substitution is located in

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https://doi.org/10.1016/j.isci.2023.107208

Table 1. Amino acid substitutions detected after passages in the presence of convalescent and/or vaccinee plasma

Pango lineage of virus	Plasma ID	NT titer[a]	Line[b]	Amino acid substitutions in the spike at the indicated passage number[c]				
				10	20	30	40	50
B.1	HICo-002	1:53	1	n.t.	n.t.	n.t.	n.t.	No mutation
			2	n.t.	n.t.	n.t.	n.t.	No mutation
			3	L141F	L141F	L141F	L141F	L141F
	HICo-004	1:80	1	A1174T	K921E and A1174T	K921E and A1174T	K921E and A1174T	K921E and A1174T
			2	Y248C	Y248C	Y248C and E619A	Y248C and E619A	Y248C and E619A
			3	No mutation	No mutation	L517F	L517F	L517F
	HICo-011	1:33	1	F140del	F140del	F140del	F140del	F140del
			2	No mutation	F140del	F140del	F140del	F140del
			3	A67V	A67V	A67V	A67V	W64G and A67V
	KYCo-001	1:20	1	n.t.	n.t.	n.t.	n.t.	No mutation
			2	n.t.	n.t.	n.t.	n.t.	No mutation
			3	No mutation	del241-243	del241-243	del241-243	del241-243
R.1	HICo-002	1:43	1	n.t.	n.t.	n.t.	No mutation	ND
			2	n.t.	n.t.	n.t.	No mutation	ND
			3	No mutation	No mutation	L176F	L176F	ND
	HICo-004	1:53	1	n.t.	n.t.	n.t.	No mutation	ND
			2	T1009I	T1009I	T1009I	V143D, Y144del, and T1009I	ND
			3	n.t.	n.t.	n.t.	No mutation	ND
	HICo-011	1:43	1	D253A	D253A	D253A	D253A	ND
			2	No mutation	No mutation	No mutation	F140del	ND
			3	No mutation	No mutation	K921Q	L5F and K921Q	ND
	KYCo-001	1:16	1	No mutation	D808N	D808N	D808N	ND
			2	No mutation	No mutation	No mutation	P1143S	ND
			3	No mutation	No mutation	No mutation	R682W and I1183V	ND

(*Continued on next page*)

Table 1. *Continued*

Pango lineage of virus	Plasma ID	NT titer[a]	Line[b]	Amino acid substitutions in the spike at the indicated passage number[c]				
				10	20	30	40	50
B.1.351	HP(H)-008	1:13	1	No mutation	No mutation	K444T	ND	ND
			2	No mutation	M177T	M177T	ND	ND
			3	No mutation	P9S and A892V	P9S and A892V	ND	ND
	HP(H)-024	1:47	1	n.t.	n.t.	No mutation	ND	ND
			2	H1101Y	H1101Y	K444N and H1101Y	ND	ND
			3	n.t.	n.t.	No mutation	ND	ND
	SUCo-85	>1:160	1	n.t.	n.t.	No mutation	ND	ND
			2	n.t.	n.t.	No mutation	ND	ND
			3	No mutation	R683W	R683W	ND	ND
	SUCo-86	1:53	1	n.t.	n.t.	No mutation	ND	ND
			2	n.t.	n.t.	No mutation	ND	ND
			3	n.t.	n.t.	No mutation	ND	ND
B.1.617.2	HP(H)-008	1:17	1	n.t.	n.t.	No mutation	ND	ND
			2	n.t.	n.t.	No mutation	ND	ND
			3	T1006A	T1006A	T1006A	ND	ND
	HP(H)-024	1:40	1	No mutation	T1006A	T1006A	ND	ND
			2	n.t.	n.t.	No mutation	ND	ND
			3	n.t.	n.t.	No mutation	ND	ND
	SUCo-85	1:107	1	S698L	S698L	S698L	ND	ND
			2	S698L	S698L	S698L	ND	ND
			3	No mutation	No mutation	S698L	ND	ND
	SUCo-86	1:67	1	P25T	P25T	P25T and T95I	ND	ND
			2	n.t.	n.t.	No mutation	ND	ND
			3	G181A	G181A	G181A	ND	ND

n.t., sequencing was not tested.

n.d., virus passages were not done.

[a]NT titer, neutralization titers against the virus prior to passaging with plasma.

[b]Lines 1–3 were three independent experiments.

[c]Amino acid sequences after passages were compared with the sequences of the pre-passage viruses.

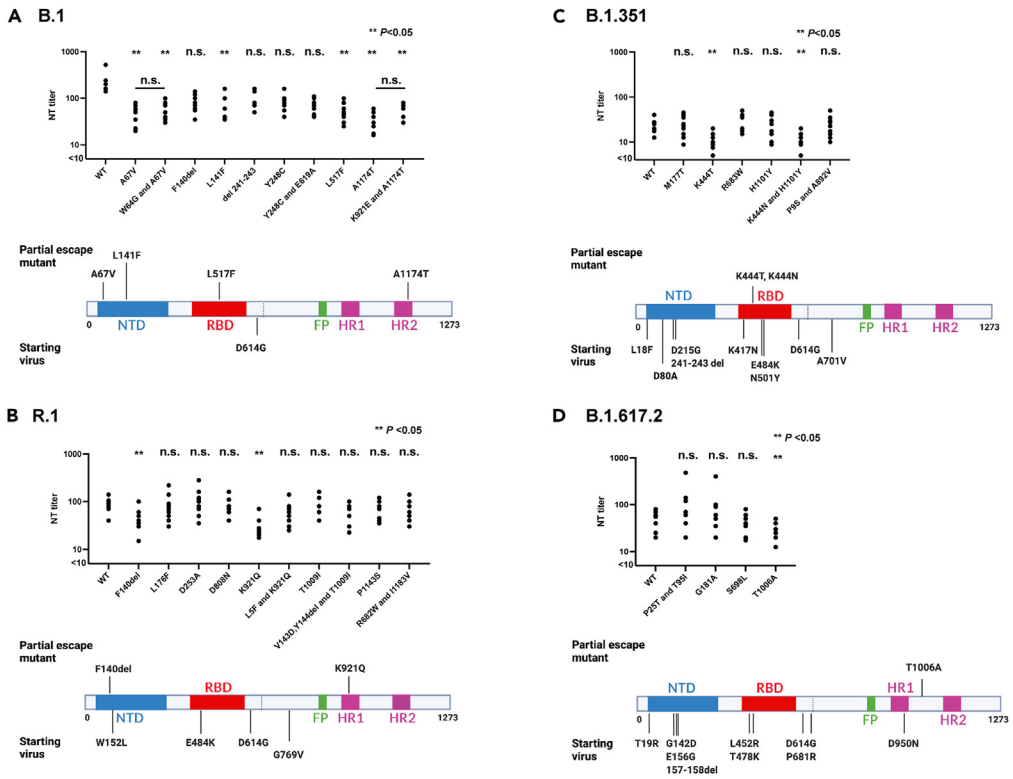

Figure 1. Neutralization titers of vaccinee plasma against mutant viruses and mapping of escape mutations

(A–D) Neutralization titers of 10 vaccinee plasma samples against wild-type and mutant viruses were measured in quadruplicate and mean titer values were plotted. B.1 virus (A), R.1 virus (B), B.1.351 virus (C), or B.1.617.2 virus (D) was used as the starting virus. Amino acid differences compared with lineage A virus and amino acid substitutions that affect the neutralization titers of vaccinee plasma samples are mapped below and above the schematic diagrams of the S protein, respectively. NTD, N-terminal domain. RBD, receptor-binding domain. FP, fusion peptide. HR, heptad repeat. Friedman's test followed by Dunn's multiple comparisons test was performed to detect any significant difference in neutralization titers. A p value of < 0.05 is considered significantly different (**); n.s., not statistically significant.

heptad repeat 1 (HR1) (Figures 1B and 2B). Among the B.1.351-derived mutant viruses, neutralization titers against the K444T and K444N plus H1101Y mutant viruses were significantly lower than those against wild-type virus (Figure 1C and Table S7). The neutralization titer against the H1101Y mutant virus was similar to that against wild-type virus. These results indicate that the K444T and K444N substitutions in the RBD affect the neutralization potency of vaccinated individuals (Figures 1C and 2C) For the B.1.617.2-derived mutant viruses, the neutralization titer against the T1006A mutant virus was significantly lower than that against wild-type virus (Figure 1D and Table S8), indicating that the T1006A substitution in the S2 region affects the neutralization potency of vaccinated individuals (Figures 1D and 2D) The detected substitutions that led to antigenic changes are summarized in Figure 3.

Reductions in neutralization titers against mutant viruses varies among individuals

The neutralization titers against mutant viruses that showed antigenic differences compared with their parent viruses (see Figures 1A and 1B) were used to calculate fold-reduction values as shown in Figure 4A. Among the mutant viruses derived from the B.1 virus, HP(H)-191, −200, −217, −218, −228, −250, and SU(H)-08 plasma samples showed the greatest reduction in neutralizing titer against the A1174T mutant but the degree of reduction in neutralizing titer against the other mutants differed among individuals: for HP(H)-200 and −217, the second largest reduction was against the A67V mutant; and for HP(H)-250 and SU(H)-08, it was against the L517F mutant; HP(H)-218 showed similar reductions against the A67V and L517F mutants (Figure 4A). The reduction in neutralization titer of HP(H)-197 against the L141F mutant was the largest, whereas HP(H)-10, −279 and SU(H)-08 showed less reduction in neutralization titers against the L141F mutant. For the mutant viruses derived from the R.1 virus, HP(H)-200 showed similar reductions against the F140del mutant and the K921Q mutant. The reduction in neutralization titers of HP(H)-217

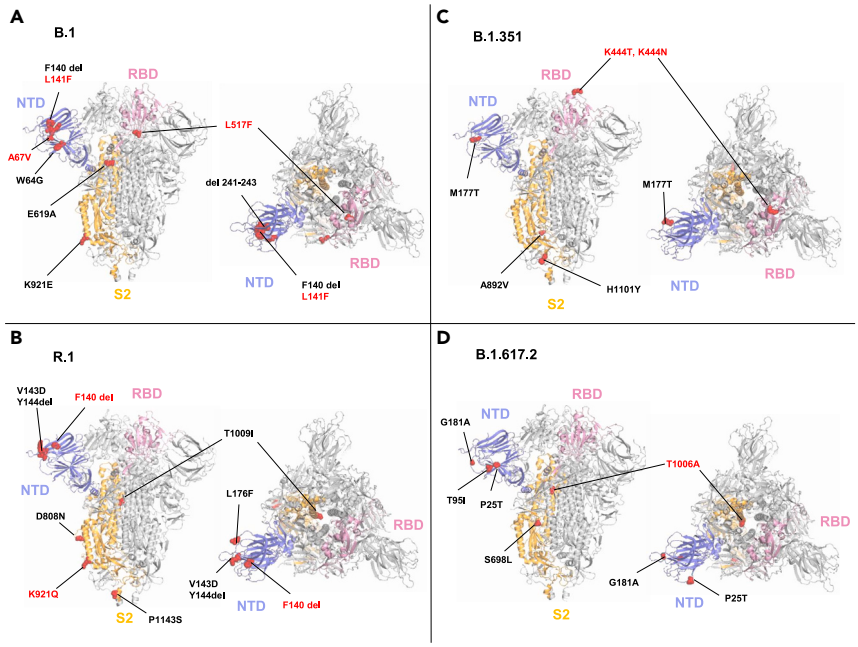

Figure 2. Mapping of amino acid substitutions on the structure of the S protein

(A–D) Amino acid substitutions that were detected after passages of B.1 virus (A), R.1 virus (B), B.1.351 virus (C), or B.1.617.2 virus (D) in the presence of plasma are mapped on the structure of the S protein (PDB: 7TGY). Amino acid substitutions that affected neutralization titers are indicated by red letters on the side (left) and top (right) views of the S protein. The amino acids substitutions L5F, P9S, Y248C, D253A, R682W, R683W, A1174T, and I1183V are not mapped because there are no structural data for these positions. The NTD and RBD and S2 are indicated in blue and pink, and orange, respectively.

against the F140del mutant was greater than those against the K921Q mutant, whereas the other plasma samples showed a greater reduction against the K921Q mutant than against the F140del mutant (Figure 4A). These results indicate that the predominant epitopes recognized by neutralizing antibodies differ among vaccinees.

We next examined the degree of escape of the mutant viruses by using the convalescent samples collected from individuals infected with A or B.1 virus (Figure 4B, Table S9). HICo-005 and -009 showed similar reductions in neutralization titers against the A67V, L517F, and A1174T viruses (Figure 4B). KOCo-080 showed similar reductions in neutralization titers against the A67V and A1174T mutants, whereas KOCo-109 showed similar reductions in neutralizing titers against the L517F and A1174T mutants. HICo-006 showed a decrease in neutralization titer against the A67V mutant but not against other mutants. Thus, the convalescent samples also revealed a difference in the epitopes recognized by individuals.

To confirm that the epitopes recognized as predominant by neutralizing antibodies differ among individuals, We compared the fold-reduction in neutralizing titers of vaccinee plasma samples against several SARS-CoV-2 variants including B.1.1.7 (alpha), B.1.351 (beta), B.1.617.2 (delta), BA.1, and BA.5 viruses (Figure 4C, Table S10). We chose plasma samples with a high neutralizing titer against the A or BA.1 virus from vaccinees who received three doses of mRNA vaccine.[33] HP(H)-197 showed similar reductions in neutralization titers against BA.1 virus and BA.5 virus, whereas the other plasma samples showed the greatest reduction in neutralization titer against BA.5 virus (Figure 4C). HP(H)-026, −106, and −216 showed similar reductions against B.1.351 (beta) and BA.1 viruses. In contrast, HP(H)-004, −019, −115, −131, −168, −193, −197, −209, and −303 showed a greater reduction against BA.1 than against B.1.351 (beta). These results suggest that the epitopes recognized as predominant by neutralizing antibodies differ among individuals, for naturally emerged variants as well.

DISCUSSION

We detected amino acid substitutions in the S protein of SARS-CoV-2 after multiple passages in the presence of human plasma collected from individuals who were infected with SARS-CoV-2 and/or

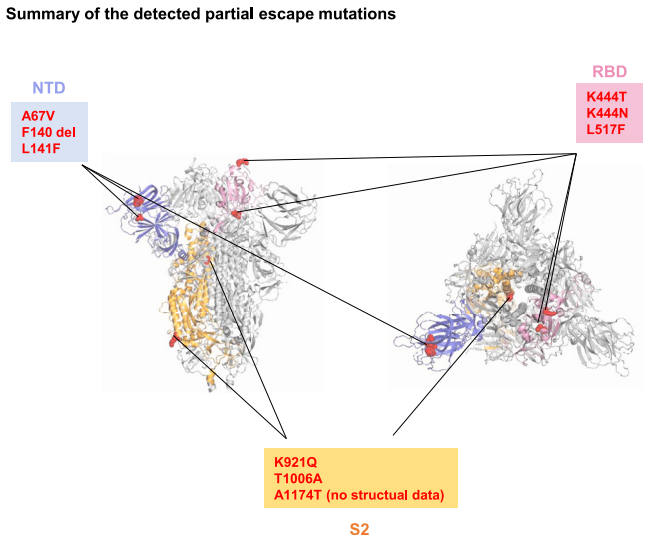

Figure 3. Mapping of amino acid substitutions on the structure of the S protein involved in antigenic changes
Summary of the amino acid substitutions involved in antigenic changes detected in this study.

received two doses of Pfizer mRNA vaccine. Although mutant viruses possessing these amino acid substitutions in the NTD, RBD, or S2 region partially escaped from neutralizing antibodies elicited by infection and vaccination, the amino acid substitutions that emerged after virus passages in the presence of plasma samples varied depending on the plasma samples and starting viruses. Furthermore, the relative neutralization potency against the mutant viruses we obtained or VOCs differed among individuals. Previous comprehensive mutational analysis using pseudo-type virus also showed that the impact of amino acid substitutions in three main epitopes in the RBD varied among individuals.[9] Many factors are likely involved in determining which neutralizing epitopes become dominant in an individual. Among them, the immunological background formed by pre-exposure history to other coronaviruses such as OC43 and HKU1 may play a role. Antibody titers against the S protein of OC43 and HKU1 are increased by SARS-CoV-2 infection and vaccination,[34,35] suggesting that cross-reactive antibodies are elicited upon exposure to the S protein of SARS-CoV-2. Therefore, the history of infection with beta coronaviruses such as OC43 and HKU1 that harbor common neutralizing epitopes to SARS-CoV-2 shapes the immunological background and might affect the antibody response against neutralizing epitopes on the S protein. It is also possible that antibody responses are controlled by the genetic background. There are several reports that germline influence on the SARS-CoV-2 neutralizing antibodies.[36,37] Further studies are required to understand how the predominant epitopes recognized by individuals are determined.

The K921Q, T1006A, and A1174T substitutions in the S2 region affected the neutralization potency of the plasma samples collected from vaccinees, indicating that these mutations contribute to escape from neutralizing antibodies. It has been reported that antibodies against the S2 region of the S protein are elicited by vaccination and SARS-CoV-2 infection,[36,38] and epitope analysis using phage-display has indicated that amino acid substitutions in the HR2 and its upstream linker region are potential targets of neutralizing antibodies.[39,40] Similarly, several human monoclonal neutralizing antibodies that recognize the HR in gp41 of human immunodeficiency virus have been reported.[41,42] Furthermore, human monoclonal antibodies that recognize the S2 region (clones CC40.8 and S2P6) were found to protect animals from SARS-CoV-2 infection, and immunization with the stem region of the S protein conferred protection against challenge infection with SARS-CoV-2.[43,44] Taken together, these reports and our results in the present study indicate that neutralizing epitopes exist in the S2 region, including HR1 and HR2 and the K921Q, T1006A, and A1174T substitutions in this region could affect the ability of neutralizing antibodies that recognize the S2 region, resulting in the escape from such antibodies.

Our results show that escape mutants emerged as a result of the A67V, F140del, and L141F substitutions in the NTD and the K444T, K444N, and L517F substitutions in the RBD. Since the deletion of F140 in the N3 loop of

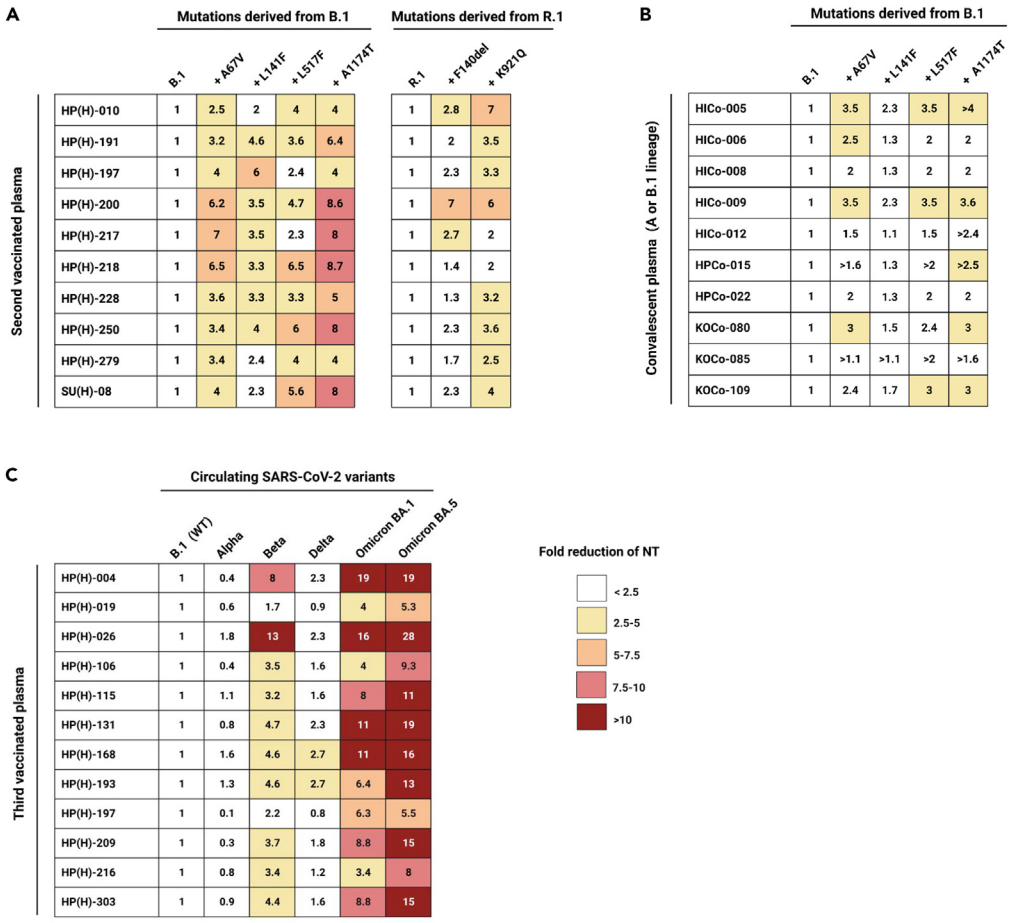

Figure 4. Reduction in neutralization titers of vaccinees or patients against mutant viruses and VOCs

The neutralization assay was performed in quadruplicate using vaccinee or convalescent plasma samples.

(A) B.1-derived mutant viruses or R.1-derived mutant viruses. The panels show the fold-changes in neutralization titers relative to the starting virus for each two-dose-vaccinated plasma samples.

(B) The fold-changes in neutralization titers of B.1-derived mutant viruses compared with the starting viruses for convalescent plasma samples.

(C) The fold-changes in neutralization titers of SARS-CoV-2 variants (B.1.1.7, B.1.351, B.1.617.2, BA.1, and BA.5) for three-dose-vaccinated plasma samples.

the NTD has been shown to reduce neutralization potency,[15] it is possible that the L141F substitution also affects the neutralization potency of vaccine-induced antibodies. The deletion of both H69 and V70 at the N2 loop of the NTD is associated indirectly with a reduction of neutralization titers.[23,45] The A67V substitution, which is located near the N2 loop of the NTD, was found in BA.1 lineage (omicron) viruses. The K444 T/N substitution is located at the 443–450 loop in the RBD, where amino acid substitutions are known to reduce the binding of antibodies.[9] The K444T substitution was found in BQ.1 and BQ1.1 subvariants.[4] To our knowledge, this is the first report of partial escape due to the L517F substitution located near the interface between the RBD and NTD based on the structural data,[46] suggesting that it may affect a ratio of open ("standing up") and closed ("lying down") conformations of S protein. It could contribute to escape from neutralizing antibody against RBD. However, the GISAID-registered database reports that 0.01% of the circulating strains sequenced carry the L517F substitution.[47] Further studies are necessary to elucidate how these substitutions contribute to escape from the neutralizing antibodies against the NTD and RBD.

VOCs such as the delta and omicron variants possess many amino acid substitutions compared with the original lineage A viruses. In the present study, we found that a limited numbers of the amino acid substitutions in the S protein were detected after 30–50 passages under constant selective pressure of neutralizing antibodies derived from vaccinated and/or infected individuals. Therefore, the enhanced accumulation of amino acid

substitutions in the S protein of VOCs is likely caused under specific circumstances such as persistent SARS-CoV-2 infection in immunocompromised patients.[28–30] Further analyses are required to predict the next variants that are likely to emerge.

In conclusion, this study identified several amino acid substitutions, including some in the S2 region, that affect the neutralizing potency of infection- or vaccine-induced antibodies. Furthermore, the epitopes recognized as predominant by neutralizing antibodies differ among individuals.

Limitation of the study

We note several limitations in our study. First, we did not use the WHO standard viral neuralization protocol. Hence, our findings involving neutralization titers cannot be compared with those of studies that used this protocol. Second, the limited number of sera in our study did not allow us to test whether differences in epitope recognition between individuals could be classified. Such information could pave the way for personalized vaccines that would best elicit high neutralization antibodies for that individual. Finally, we could not obtain antigenic mutant viruses that resembled omicron variants even after 50 passages with the antisera.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107208.

ACKNOWLEDGMENTS

We thank Atsuhiro Yasuhara, Ryuta Uraki, Tadashi Maemura, Hiroshi Ueki, Maki Kiso, Masaki Imai, Kyoko Yokota, and Tomoka Nagashima for assistance with experiments; Shohei Matsubara and Kazuhiko Ikeuchi for collecting plasma samples, and Susan Watson for editing the manuscript. This work was supported by a Grant-in-Aid for JSPS Research Fellows (22J11603) and the Japan Agency for Medical Research and Development (JP19fk0108166, JP22wm0125002, and JP223fa627001). The funder of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report.

AUTHOR CONTRIBUTIONS

S. Yamamoto., S. Yamayoshi., and Y.K. designed the study, analyzed the data, and wrote the manuscript. S. Yamamoto performed the escape mutant work, titrated virus, sequenced virus. S. Yamamoto and M.I. performed the neutralizing assays. S. Yamayoshi performed the enzyme-linked immunosorbent assay. I.N., R.B., S.K., T.O., S.H., H.K., H. Nakajima., Y.U., K.Y., N.S., H. Nagai., M.S., E.A., M.K., T.T., H.Y. collected the human blood and virus samples. C.D., M.O., J.M., Y.F., M.U., K.H., Y.T. performed the collection and/or processing of clinical samples. All authors reviewed and approved the manuscript.

DECLARATION OF INTERESTS

Y.K. has received unrelated funding support from the Scientific Advisory Boards of Moderna and Immunome, Carnival Corporation, Senda Biosciences, and Vir Biotechnology. Fuji rebio, Shinya corporation, Kyoritu Seiyaku, KM Biologics, Otsuka Pharmaceutical, Shinogi and Co. LTD, Tauns Laboratories, Toyama Chemical, and Daiichi Sankyo Pharmaceutical. The other authors have no conflicts of interest to declare.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

Received: February 15, 2023
Revised: April 21, 2023
Accepted: June 21, 2023
Published: June 25, 2023

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
SARS-CoV-2/UT-HP095-1N/Human/2020/Tokyo	Halfmann et al.[48]	B.1 lineage
SARS-CoV-2/UT-HP123-1Nf/Human/2021/Tokyo	This Paper	R.1 lineage
SARS-CoV-2/UT-HP127-1Nf/Human/2021/Tokyo	Takashita et al. [49]	B.1.1.7 lineage
hCoV-19/USA/MD-HP01542/2021	Takashita et al.[49]	B.1.351 lineage
SARS-CoV-2/TKYTK1734/Human/2021/Tokyo	Saito et al.[50]	B.1.617.2 lineage
hCoV-19/Japan/NC928-2N/2021	Takashita et al.[49]	BA.1 lineage
SARS-CoV-2/human/USA/COR-22-063113/2022	Cheng et al.[51] Uraki et al.[33,52]	BA.5 lineage
Biological samples		
Convalescent plasmas	IMSUT Hospital, Yokohama City University School of Medicine, Keiyu Hospital, Keio University School of Medicine	N/A
Plasma samples collected from 2-doses mRNA vaccinees	IMSUT Hospital Saiseikai Utsunomiya Hospital	N/A
Plasma samples collected from 2-doses mRNA vaccinees who had been infected with SARS-CoV-2	Saiseikai Utsunomiya Hospital	N/A
Plasma samples collected from 3-doses mRNA vaccinees	Uraki et al.[33,52]	N/A
Chemicals, peptides, and recombinant proteins		
BigDye Terminator v3.1 cycle sequencing kit	Applied Biosystems	Cat #4337455
Dulbecco's Modified Eagle Medium (high glucose)	SIGMA	Cat #D5796
Fetal Bovine Serum	gibco	Cat #10437-028
G418 solution	Invivo Gen	Cat # ant-gn-5
Penicillin-Streptomycin Solution	FUJIFILM Wako Pure Chemical Corporation	Cat #168-23191
Plasmocin	Invivo Gen	Cat #ant-mpp
Critical commercial assays		
ABI 3130xl sequencer	Applied Biosystems	N/A
Mini elute Gel Extraction	QIAGEN	Cat #No 28606
QIAmp Viral RNA Mini Kit	QIAGEN	Cat #No 52904
Deposited data		
The structure of the SARS-CoV-2 Spike protein	PyMOL	PDB: 7TGY
Experimental models: Cell lines		
VeroE6/TMPRSS2 cells	JCRB Cell Bank	JCRB1819

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Primer 20000F: (5′-TCACTGTCTT TTTTGATGGT AGAGT-3′)	FASMAC	N/A
Primer 25500R: (5′-TTATCTAACT CCTCCTTGAA TGA-3′)	FASMAC	N/A
Software and algorithms		
ATGC ver.7	GENETYX	https://www.genetyx.co.jp/products/atgc_7/index.html
BioRender	BioRender.	https://biorender.com
GraphPad Prism 9.3.0	GraphPad Software, Inc.	https://www.graphpad.com/scientific-software/prism/

RESOURCE AVAILABILITY

Lead contact

Further information regarding the findings of this study and requests for resources should be directed to and will be fulfilled by the lead contact, Yoshihiro Kawaoka (yoshihiro.kawaoka@wisc.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request. Additional Supplemental Items are available from Mendeley Data at https://doi.org/10.17632/44dwzc4cyt.1.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics and biosafety statement

Because studies of the selection of escape mutant viruses by passaging SARS-CoV-2 in the presence of convalescent or vaccinated plasma are considered gain-of-function studies and therefore we sought and received an approved by the Research Microbiology Safety Committee of the Institute of Medical Science, The University of Tokyo before the study was initiated. The results and this manuscript were also reviewed and approved for the submission by the committee. All experiments with authentic SARS-CoV-2 were performed in enhanced biosafety level 3 (BSL3) containment laboratories at the University of Tokyo (Tokyo, Japan). All experiments were conducted by following regulations set forth by the Ministry of Agriculture, Forestry and Fishers, Japan and Ministry of Education, Culture, Sports, Science and Technology, Japan.

All protocols collecting specimens from human subjects were reviewed and approved by the Research Ethics Review Committee of the Institute of Medical Science, at the University of Tokyo (2019-71-0201, 2020-74-0226). Samples were collected by following protocols approved by the ethics Review Committee of the Institute of Medical Science, at the University of Tokyo. Informed consent was obtained from all individuals.

Cells

VeroE6/TMPRSS2 cells[53] were maintained in Dulbecco's Modified Eagle Medium (high glucose) supplemented with 10% FBS, Penicillin-Streptomycin Solution, G418 solution, and Plasmocin at 37°C and 5% CO_2 and were regularly tested for mycoplasma.

Viruses

SARS-CoV-2 lineage B.1 (SARS-CoV-2/UT-HP095-1N/Human/2020/Tokyo), R.1 (SARS-CoV-2/UT-HP123-1Nf/Human/2021/Tokyo), B.1.1.7 (alpha variant, SARS-CoV-2/UT-HP127-1Nf/Human/2021/Tokyo), B.1.351

(beta variant, hCoV-19/USA/MD-HP01542/2021), B.1.617.2 (delta variant, SARS-CoV-2/TKYTK1734/Human/2021/Tokyo), and B.1.1.529 (omicron variant BA.1, hCoV-19/Japan/NC928-2N/2021, omicron BA.5, SARS-COV-2/human/USA/COR-22-063113/2022) were used.[48–52] In the S protein, the B.1 virus possessed the D614G substitution, the R.1 virus possessed the W152L, E484K, D614G, and G769V substitutions, the alpha variant possessed the del 69-70, del 144, N501Y, A570D, D614G, P681H, T716I, S982A, and D1118H substitutions, the beta variant possessed the L18F, D80A, D215G, del241-243, K417N, E484K, N501Y, D614G, and A701V substitutions, the delta variant possessed the T19R, G142D, E156G, del157-158, L452R, T478K, D614G, P681R, and D950N substitutions, and the omicron BA.1 variant possessed the A67V, del69-70, T95I, G142D, del143-145, N211del, L212I, ins214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, and L981F substitutions. The BA.5 virus possessed the T19I, del24-26, A27S, del69-70, T76I, G142D, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, L452R, S477N, T478K, E484A,F486V, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, and N969K substitutions. All viruses were isolated and propagated in VeroE6/TMPRSS2 cells.

Human plasma samples

The human plasma samples were obtained from individuals who recovered from infection with SARS-CoV-2 lineage A or B.1 or from individuals who received two doses of Pfizer mRNA vaccine (BNT162b2) three weeks before at IMSUT hospital, Keiyu hospital, Yokoyama city university hospital, Keio University Hospital, or Saiseikai utsunomiya hospital. The human plasma samples obtained from individuals who received three doses of Pfizer mRNA vaccine (BNT162b2) were described previously.[33]

METHOD DETAILS

Neutralization assay

Plasma samples were initially 4- to 10-fold diluted and then serially two-fold diluted of plasma samples. The diluted plasma samples (50 μL) were mixed with 200-400 $TCID_{50}$ of the indicated viruses in 50 μL of culture media and incubated for 1 h at room temperature. The 100 μL of virus-plasma mixture was inoculated into VeroE6/TMPRSS2 cells in a 96-well plate after removal of the culture medium and incubated for 1 h at 37°C. Fresh culture medium containing 5% FBS (100 μL/well) was added before incubation at 37°C for 3 days. Cytopathic effect (CPE) was confirmed by observation under an inverted microscope. Neutralization titer for each experiment was determined as the highest plasma dilution that prevented the appearance of CPE. When the CPE was observed in the well of a10-fold diluted plasma sample, we considered it 1:5 for the calculation of the neutralization titer. The neutralization titers of each plasma sample were calculated as the mean of quadruplicate or triplicate examinations. For Tables S1 and S4, 25 μL of the diluted plasma samples were mixed with 100-200 $TCID_{50}$ of virus in 25 μL media, and total 50 μL of virus-plasma mixture were inoculated into the cells. The neutralization titers of plasma sample were determined according to the lower neutralization titer of each experiment in duplicates examinations.

Virus passages in the presence of convalescent and/or vaccinee plasma samples

The indicated lineages of SARS-CoV-2 (100-200 $TCID_{50}$ /25 μL) were passaged in the presence of serially 10-fold diluted convalescent and/or vaccinee plasma samples. Cell culture supernatant containing viruses was harvested from the CPE-positive well with the lowest plasma dilution and then used for the next passage. The harvested viruses were simply diluted 100-100,000 fold without virus titration and mixed with diluted plasma samples. This virus passage was performed every three days. This procedure was repeated 30−50 times.

Sequence analysis

Nucleotide sequences of the S gene of the passaged viruses were determined by use of standard Sanger sequencing. Briefly, viral RNA was extracted by using a QIAamp viral RNA mini kit (QUIAGEN). cDNA was synthesized by reverse transcription using an oligo dT primer. PCR was performed using primers 20000F (5′-TCACTGTCTT TTTTGATGGT AGAGT-3′) and 25500R (5′-TTATCTAACT CCTCCTTGAA TGA-3′). The PCR product was purified by using a Mini elute Gel Extraction Kit and then sequenced by using a

BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) on an ABI 3730xl sequencer (Applied Biosystems). Acquired sequences were assembled by using ATGC ver.7 (Sequence assembly software, GENETYX).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Friedman's test with Dunn's post-hoc correction for multiple comparisons (GraphPad Prism 9.3.0) was performed to calculate the *P*-values of neutralizing assays.