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BRIEF REPORT

Anti-membrane Antibodies Persist at Least One Year and Discriminate Between Past Coronavirus Disease 2019 Infection and Vaccination

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Background. The consequences of past coronavirus disease 2019 (COVID-19) infection for personal and population health are emerging, but accurately identifying distant infection is a challenge. Anti-spike antibodies rise after both vaccination and infection and anti-nucleocapsid antibodies rapidly decline.

Methods. We evaluated anti-membrane antibodies in COVID-19 naive, vaccinated, and convalescent subjects to determine if they persist and accurately detect distant infection.

Results. We found that anti-membrane antibodies persist for at least 1 year and are a sensitive and specific marker of past COVID-19 infection.

Conclusions. Thus, anti-membrane and anti-spike antibodies together can differentiate between COVID-19 convalescent, vaccinated, and naive states to advance public health and research.

Keywords. antibody; COVID-19; SARS-CoV-2.

The cardinal features and challenges of the coronavirus disease 2019 (COVID-19) pandemic have changed. The pandemic was initially defined by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections in an immunologically naive population. Now, immunity from vaccination, infection, or both is common, reducing the severity of future infection waves. However, identifying infection for research and public health efforts is limited by the need to perform viral testing during acute infection (which may not occur during asymptomatic disease or pandemic surges that deplete resources) and by shortcomings in serologic testing [1]. Anti-nucleocapsid antibodies often decline to seronegativity just months after infection [2–4]. Anti-spike antibodies persist at least 1 year postinfection [5, 6] but typically cannot differentiate between infection and vaccination [7–9]. Anti-membrane antibodies develop soon after SARS-CoV-2 infection [10–12], but they are rarely assessed and their longevity is unknown. In this study, we evaluated antibodies against the receptor-binding domain (RBD) of spike, nucleocapsid, and membrane antigens in naive, COVID-19 vaccinated, and COVID-19 convalescent subjects up to 1 year postsymptom resolution to evaluate the persistence of anti-membrane antibodies and to identify antigens that discriminate between distant infection, vaccination, and naive states.

METHODS

Human Subjects

Human studies were approved by the University of Wisconsin (UW) Institutional Review Board and human subjects provided written informed consent. Sera and data collected before 2019 from 60 COVID-19 naive adults without inflammatory disease (exception: 1 subject with psoriatic arthritis using adalimumab to match 2 COVID-19 convalescent subjects using adalimumab) were obtained from the UW Rheumatology Biorepository [13].

Coronavirus disease 2019 convalescent sera and data were obtained from the UW COVID-19 Convalescent Biorepository [14]. In brief, in the spring of 2020, adults with a positive SARS-CoV-2 polymerase chain reaction (PCR) test at UW Health were invited to participate until 121 subjects were recruited. Demographic and clinical information were collected by questionnaire and electronic medical record (EMR) abstraction. Coronavirus disease 2019 severity was diverse: mild (n = 12), moderate (n = 86), severe (n = 15), and critical (n = 8) as previously defined [14]. Subjects provided blood and clinical information 5 weeks (n = 121), 3 months (n = 115), 6 months (n = 98), and 12 months (n = 100) ±3 weeks after symptom resolution. One sample collected >3 weeks from the 3-month timepoint and subjects for whom the 5-week timepoint was collected >3 weeks from the intended timepoint (n = 1) or missed >1 blood draw (n = 16) were excluded from comparative analyses, generating sample sizes of 104 (5 weeks), 101 (3 months), 97 (6 months), and 98 (12 months). Based on anti-RBD immunoglobulin (Ig) elevation timing postvaccination (Supplementary Figure 1), COVID-19 convalescent subjects at 12 months were considered vaccinated if they received 1 vaccine dose ≥5 days before sample collection (n = 77) and unvaccinated (n = 21) if they

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received no vaccine (n = 17) or their first or only vaccine dose <5 days before sample collection (n = 4).

Vaccinated individuals without past COVID-19 (n = 21) were recruited by flyers at UW Health in summer of 2021. Complete vaccination (>3 weeks after 2 messenger ribonucleic acid [mRNA] vaccine doses or 1 Ad26.COV2.S dose) and lack of known COVID-19 was confirmed by questionnaire and EMR review.

Limited clinical data, sera, and SARS-CoV-2 lineages were provided by UW Health Infection Control for 20 completely vaccinated healthcare workers with breakthrough COVID-19 (positive PCR and symptoms) in the spring of 2021. Blood collection occurred ~1 day after SARS-CoV-2 PCR (range, 0–4 days) and ~3 days after symptom onset (range, 0–5 days). Four breakthrough cases also had PCR-positive COVID-19 before vaccination completion and 3–6 months before breakthrough infection. Healthcare workers with breakthrough infections were invited to participate in the longitudinal study, and 3 provided blood ~8 weeks (range, 37–70 days) after the initial collection.

Anti-Receptor-Binding Domain Immunoglobulin Immunoassay

Anti-RBD Ig was detected by Lumit SARS-CoV-2 Immunoassay (Promega, Madison, WI) according to kit instructions using a TEMPEST Liquid Handler (Formulatrix, Bedford, MA) and a PHERAstar FS plate reader (BMG Labtech, Ortenberg, Germany). Sera were diluted 1:10 to use the kit's recommended sample/calibrator cutoff of 1 for seropositivity. At 1:10, the highest anti-RBD Ig values were above the linear range, but results were overall similar to a 1:200 dilution (Supplementary Figure 2), at which higher values were within the linear range.

Anti-membrane and Anti-Nucleocapsid Immunoglobulin G Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assays (ELISAs) were performed as previously described to detect IgG against SARS-CoV-2 membrane (aa 8–23, ITVEELKKLLEQWNL V-K-biotin) and nucleocapsid (aa 390–405, QTVTLLPAADL DDFSK-K-biotin) peptides [10] with the following modifications: blocking for >2.5 instead of 1 hour and serum dilution of 1:50 (nucleocapsid) or 1:500 (membrane), instead of 1:200 to maximally utilize the linear range. Relative absorbance values (IgG binding to uncoated wells subtracted from coated wells for each subject and values normalized across plates using a serum standard) of 0 were plotted as 0.0001 to allow a log scale for graphs.

Statistics

Statistical analyses were performed using Prism (GraphPad, San Diego, CA) and JMP (SAS Institute, Cary, NC) software. Antibody levels were compared between naive or vaccinated subjects versus all other groups by Kruskal-Wallis One-Way ANOVA with Dunn's multiple comparisons test. Antibody levels in unvaccinated versus vaccinated 12-month convalescent samples were compared by Mann-Whitney *U* test. Matched antibody levels across multiple timepoints in convalescent subjects were compared by Friedman test with Dunn's multiple comparisons test. Antibody levels in breakthrough infection subjects were compared at ~3 days postsymptom onset versus ~8 weeks later by Wilcoxon signed-rank test. Antibody positivity was compared between 2 groups with Fisher's exact test and among multiple groups with a χ^2 test. $P < .05$ were considered significant.

RESULTS

We quantified antibodies against RBD, nucleocapsid, and membrane antigens in sera from the following subjects: naive, vaccinated with no known COVID-19 infection, COVID-19 convalescent with sera collected 5 weeks, 3 months, 6 months, and 12 months postsymptom resolution (all initially unvaccinated), and vaccinated with subsequent SARS-CoV-2 breakthrough infection. Clinical and demographic information is in Supplementary Table 1.

For anti-RBD Ig, the chemiluminescent assay had an area under the receiver operator curve (AUC) of 0.973, 90% sensitivity, and 97% specificity (Supplementary Figure 3A), comparable to other assays [14]. Convalescent and vaccinated individuals had significantly higher anti-RBD Ig than naive subjects, with no significant difference in antibody levels or percentage seropositivity between vaccinated subjects and unvaccinated convalescent subjects (Figure 1A and 1B). In a matched analysis of the 88 subjects who provided serum at all 4 timepoints (Figure 1C), anti-RBD Ig levels were statistically different between timepoints, but the extremely small difference in medians seems unlikely to be biologically meaningful. Furthermore, the percentage of seropositive subjects at 5 weeks (91%) versus 6 months (88%) was not significantly different (Figure 1B). Because only 21 convalescent subjects remained unvaccinated at 12 months, this timepoint was not compared to the 5-week timepoint. Not surprisingly, anti-RBD Ig levels in 12-month convalescent subjects were significantly higher for those who received at least 1 dose of a vaccine compared with no vaccine (Figure 1A and Supplementary Figure 1B). Finally, all vaccinated subjects, with or without past or breakthrough infections, were seropositive for anti-RBD Ig, with no significant differences in antibody levels between vaccinated subjects with or without breakthrough infections (Figure 1A and 1B). Overall, these data suggest that anti-RBD antibodies are detectable in the vast majority of vaccinated and convalescent subjects at least 1 year after infection, but anti-RBD antibodies cannot differentiate between past infection, vaccination, and vaccination with breakthrough infection.

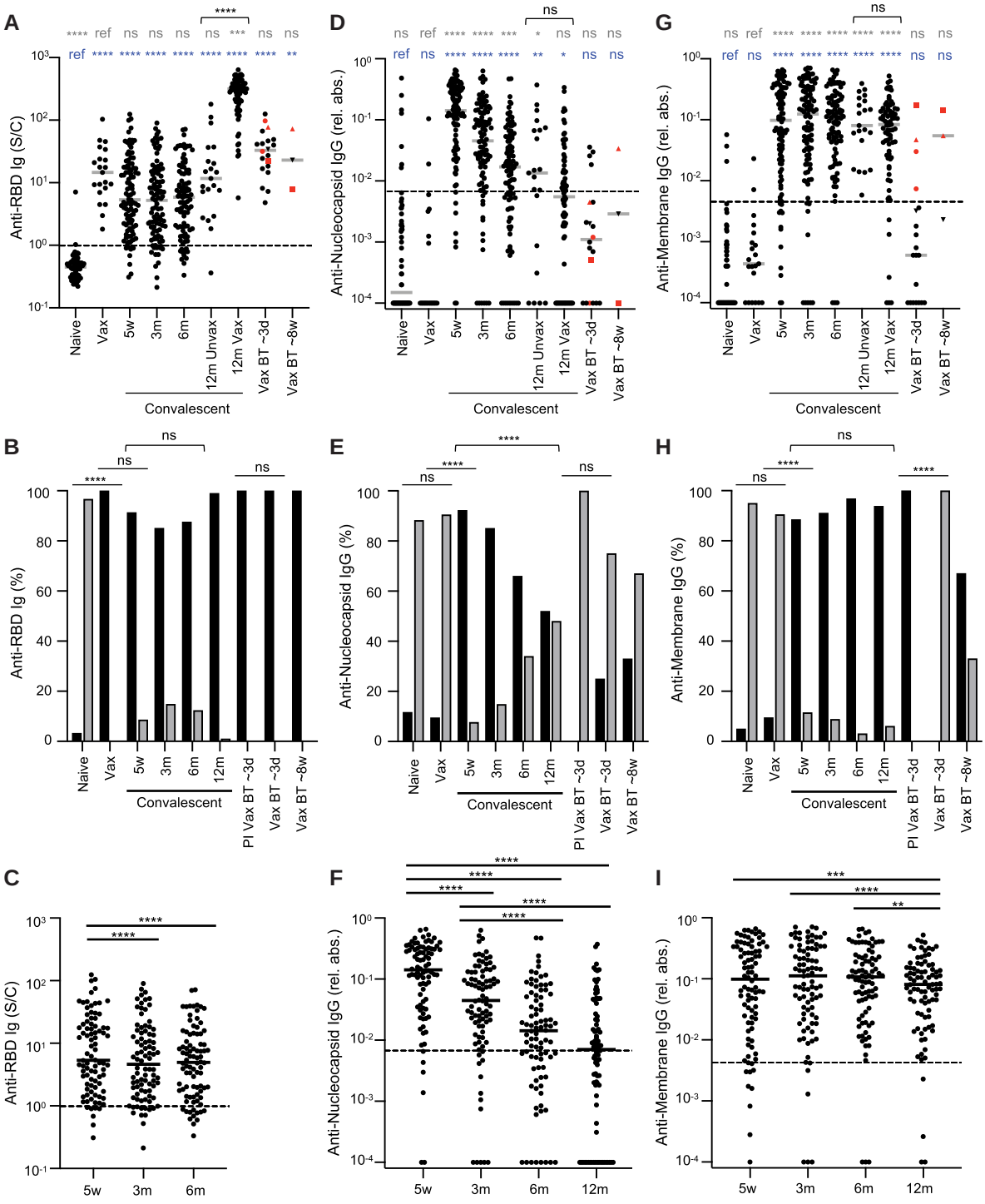

Figure 1. Anti-receptor-binding domain (RBD), anti-nucleocapsid, and anti-membrane antibodies after coronavirus disease 2019 (COVID-19) vaccination and infection. Anti-RBD immunoglobulin (Ig) was detected by immunoassay (reported as sample/calibrator [S/C]), and anti-nucleocapsid and anti-membrane IgG were quantified by enzyme-linked immunosorbent assay (reported as relative absorbance [rel. abs.]) in sera from the following subjects: naive (n = 60), vaccinated with no known COVID-19 infection (Vax, n = 21), COVID-19 convalescent 5 weeks (5w, n = 104), 3 months (3m, n = 101), 6 months (6m, n = 97), and 12 months (12m, n = 98) postsymptom resolution either vaccinated (12m Vax, n = 77) or not (12m Unvax, n = 21), vaccinated with breakthrough COVID-19 ~3 days (Vax BT ~3d, n = 20), and ~8 weeks (Vax BT ~8w, n = 3) after symptom onset including 4 subjects with previous COVID-19 infection (PI). Anti-RBD Ig (A), anti-nucleocapsid IgG (D), and anti-membrane IgG (G) levels for all groups were graphed and compared with naive (blue) or Vax (gray) by Kruskal-Wallis and Dunn's multiple comparisons tests and 12m Unvax was compared with 12m Vax by Mann-Whitney test (brackets). Vax BT with both ~3d and ~8w timepoints are represented with triangles or squares and Vax BT subjects with PI in red symbols. Percentage positive (black) and negative (gray) for anti-RBD Ig (B), anti-nucleocapsid IgG (E), and anti-membrane IgG (H) were graphed and compared between selected groups by Fisher's exact (line) or χ^2 (bracket encompassing compared groups) tests. Matched anti-RBD Ig (C), anti-nucleocapsid IgG (F), and anti-membrane IgG (I) levels were compared across timepoints for COVID-19 convalescent subjects (n = 88) by Friedman test with Dunn's multiple comparisons test. For top and bottom panels, bars indicate medians and dashed lines indicate cutoffs. For all panels, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$, or not significant (ns).

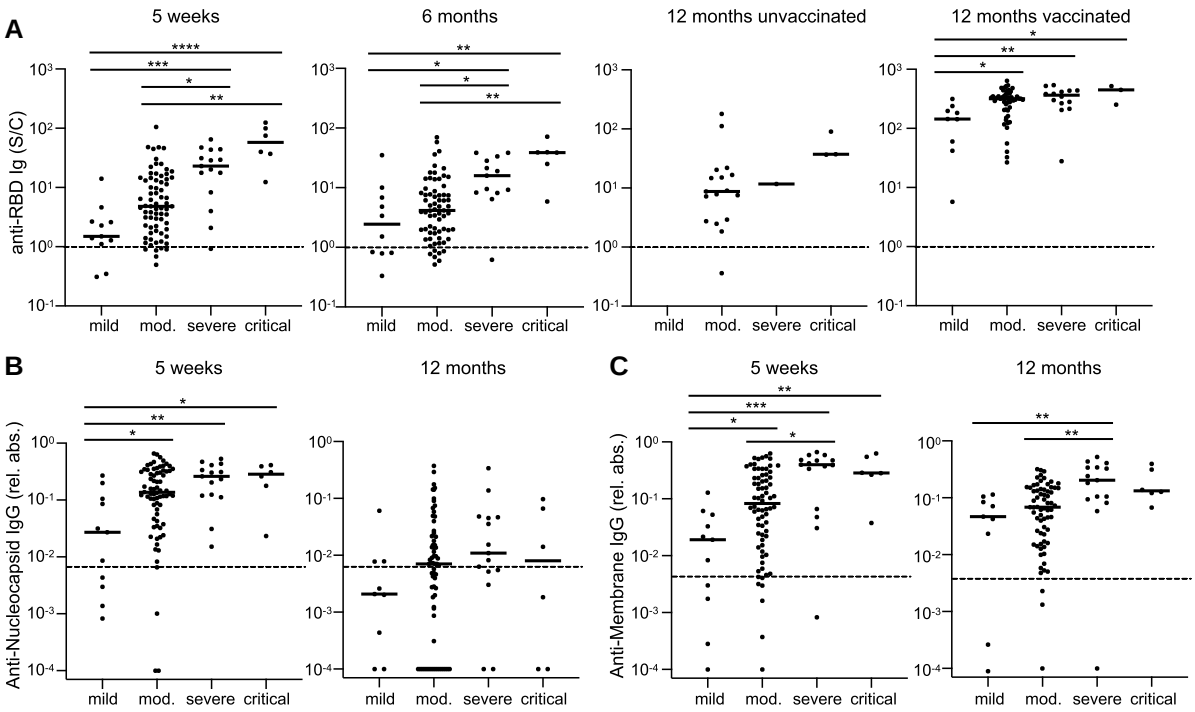

Figure 2. Anti-severe acute respiratory syndrome coronavirus 2 antibody levels are higher after more severe coronavirus disease 2019 (COVID-19). Anti-receptor-binding domain (RBD) immunoglobulin (Ig) (A), anti-nucleocapsid IgG (B), and anti-membrane IgG (C) were compared across disease severity groups by Kruskal-Wallis with Dunn's multiple comparisons test at indicated time points (mild: n = 11 5 weeks [w], n = 10 6 months [m], n = 9 12 m; moderate (mod.): n = 72 5 w, n = 68 6 m and 12 m; severe: n = 15 5 w, n = 13 6 m, n = 15 12 m; critical: n = 6 5 w, 6 m, and 12 m). For all panels, bars represent medians, dashed lines indicate cutoffs, *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$. rel. abs., relative absorbance; S/C, sample/calibrator.

Next, we evaluated anti-nucleocapsid IgG. The ELISA had an AUC of 0.919, 91% sensitivity, and 88% specificity (Supplementary Figure 3B). The low specificity of this test is consistent with other anti-nucleocapsid tests, likely due to cross-reactivity with common cold coronavirus nucleocapsid [14, 15]. Nonetheless, as expected, there was no difference in anti-nucleocapsid IgG levels between naive versus vaccinated subjects or between 12-month convalescent vaccinated versus unvaccinated subjects (Figure 1D). In addition, as expected, compared to either vaccinated or naive subjects, anti-nucleocapsid IgG levels were higher in subjects with past infection (Figure 1D). However, in a matched analysis of convalescent subjects, anti-nucleocapsid IgG levels fell significantly over time (Figure 1F) with 34% of subjects seronegative by 6 months and 48% by 12 months, a significant increase in seronegativity compared to 8% at 5 weeks (Figure 1E). It is interesting to note that none of the 4 breakthrough cases who also had COVID-19 before vaccination were seropositive for anti-nucleocapsid IgG at the time of breakthrough infection, and only 1 of 3 subjects was seropositive 8 weeks after breakthrough infection (Figure 1E). Taken together, these data highlight the rapid decline of anti-nucleocapsid antibodies.

Last, we evaluated anti-membrane IgG. The ELISA had an AUC of 0.956, 88% sensitivity, and 95% specificity (Supplementary Figure 3C). As expected, anti-membrane IgG levels and percentage seropositivity did not differ between naive and vaccinated subjects (or between 12-month convalescent vaccinated and unvaccinated subjects), but levels were significantly higher in convalescent subjects (Figure 1G and 1H). In a matched analysis over time (Figure 1I), anti-membrane IgG levels remained stable at 6 months with an extremely small decline at 12 months. However, at 12 months, 94% of convalescent samples were seropositive for anti-membrane IgG, compared to 88% at 5 weeks (Figure 1H). It is interesting to note that all 4 vaccine breakthrough infection subjects with prior COVID-19 and no breakthrough subjects without prior COVID-19 were seropositive for anti-membrane IgG during acute infection (Figure 1H). Taken together, these data demonstrate that anti-membrane IgG persists at least 1 year and can be a sensitive and specific marker of past COVID-19 infection.

Finally, we compared antibody levels at 5 weeks and 12 months post-COVID-19 across disease severity groups. As expected [10, 14], levels of all 3 antibodies were generally higher in subjects with more severe COVID-19 at both timepoints, except anti-nucleocapsid IgG at 12 months (Figure 2).

DISCUSSION

In this study, in addition to confirming that anti-RBD antibodies last at least 1 year and anti-nucleocapsid IgG declines over months [2–6], we demonstrate that anti-membrane IgG is present in the vast majority of COVID-19 convalescent patients and persists at least 1 year. Our findings are consistent with findings for IgG against a recombinant membrane antigen (polypeptide of aa 1–19 and 101–222) in the early convalescent period [11]. In contrast, Jörrißen et al [12] found that only ~20% of nonhospitalized COVID-19 convalescent subjects had IgG against a membrane peptide in the early convalescent period. Our nonhospitalized subjects alone were 88% positive for anti-membrane IgG at 5 weeks (n = 83) and 94% at 12 months (n = 77) post-COVID-19. This discrepancy may be due to their smaller sample size (n = 30) or use of a different peptide (aa 1–20).

Given the absence of anti-RBD and anti-membrane antibodies in naive subjects, the presence of only anti-RBD antibodies in vaccinated subjects, and the presence of both in COVID-19 convalescent subjects up to 12 months after infection, our study suggests that a combination of anti-RBD and anti-membrane antibody testing could be used to detect past COVID-19 infection and vaccination at a population and individual level. An analogous testing strategy for hepatitis B uses anti-surface antibodies to detect past infection or vaccination and anti-core antibodies to detect past infection. Although SARS-CoV-2 does not seem to cause persistent infection like hepatitis B, the long-term consequences of COVID-19 are still emerging, and revealing a previously undetected infection may prove to be important. At minimum, detecting unknown past infections may relieve personal anxiety about future infections in some individuals. Moreover, accurate assessment of past infection in a population could enhance the prediction of and interpretation of COVID-19 surge outcomes and inform public health policy.

Limitations of this study include that samples were collected only up to 12 months post-COVID-19 and that we quantified IgG, not IgM or IgA, that binds membrane and nucleocapsid peptides versus total Ig that binds RBD. In addition, subjects were infected by ancestral SARS-CoV-2 lineages (early 2020) or alpha and delta variants (breakthrough infections) (Supplementary Figure 4), whereas the omicron variant has a single amino acid difference in the membrane peptide (ITVEELKKLLE**E**WNLV). Finally, sample sizes for breakthrough infections were small with samples collected ~3 days after symptom onset, possibly allowing an early antibody response. Future studies are needed to evaluate later timepoints, multiple antibody isotypes, larger cohorts, and antibodies after omicron infections.

Nonetheless, we demonstrate that anti-membrane antibodies persist at least 1 year and, together with anti-RBD antibodies, can accurately identify past COVID-19 infection and vaccination.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. M. F. A., D. H. O., and M. A. S. are listed as inventors on a patent filed related to this study (PCT/US2021/051143; IDENTIFICATION OF SARS-COV-2 EPITOPES DISCRIMINATING COVID-19 INFECTION FROM CONTROL AND METHODS OF USE). Promega provided Lumit SARS-CoV-2 Immunoassay kits. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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