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RESEARCH ARTICLE

Longitudinal Serological Surveillance for COVID-19 Antibodies after Infection and Vaccination

Christopher McGee,[a] Min Shi,[b] John House,[b] Anna Drude,[a] Gladys Gonzalez,[a] Negin Martin,[c] Shih-Heng Chen,[c] Heidi Rogers,[a] Alex Njunge,[a] Xiomara Hodge,[a] Brittany Mosley,[a] Margaret George,[a] Ruhani Agrawal,[a] Catherine Wild,[a] Cynthia Smith,[a] Audrey Brown,[a] Lisa Barber,[a] Stavros Garantziotis[a]

[a]Clinical Research Branch, Division of Intramural Research, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA
[b]Biostatistics & Computational Biology Branch, Division of Intramural Research, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA
[c]Viral Vector Core, Division of Intramural Research, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA

Christopher McGee and Min Shi contributed equally as first authors. Author order was determined both alphabetically and in order of increasing seniority.

ABSTRACT The impact of COVID-19 is still felt around the world, and more information is needed regarding infection risk, vaccination responses, and the timing of booster vaccinations. We aimed to evaluate the association of vaccination with closely followed, longitudinal antibody titers and COVID-19 infection events. We conducted a natural history study in a convenience cohort in an ambulatory research unit. We measured anti-nucleocapsid and anti-spike antibody levels every 3 months for 1 year and captured weekly reports of medically confirmed COVID-19 infections. We analyzed the association of antibody titers with infection events as well as the association of the decision to receive vaccination with social, medical, and behavioral characteristics. 629 subjects were followed for 1 year, and 82.8% of them were vaccinated. 90 cases of medically confirmed COVID-19 infection were reported. Notable findings from our study include: an association of vaccination choice with social distancing, a qualitatively different anti-spike response in participants receiving the Ad26.COV2.S vaccine compared to those receiving mRNA vaccines, a muted anti-nucleocapsid response in breakthrough infections compared to unvaccinated infections, and the identification of a low antibody titer threshold associated with the risk of breakthrough infections. We conclude that, in a real-life setting, vaccination and social distancing behavior are positively correlated. The observed effect of vaccination in preventing COVID-19 may include both vaccine-mediated protection and the associated more cautious behavior exhibited by vaccinated individuals. In addition, we identified an antibody threshold associated with breakthrough infections in mRNA vaccinees, and this threshold may be used in medical decision-making regarding the timing of booster vaccinations. Therefore, our data may aid in the refinement of vaccination strategies during the COVID-19 pandemic.

IMPORTANCE The COVID-19 pandemic continues to impact societies and health care systems worldwide and is continuously evolving. Immunity via vaccination or prior infection is the first and most important line of defense against COVID-19. We still do not have complete information on how vaccination-induced or infection-induced antibody titers change with time or on how this information can be used to guide decisions regarding booster vaccination. In a longitudinal observational study of a cohort of 629 subjects, 82% of breakthrough infections in vaccinees occurred when their anti-spike antibody titers were below 3,000 AU/mL. Our findings suggest that there may be an antibody threshold associated with breakthrough infections and that this threshold could possibly be used to aid decision-making regarding booster vaccinations. In addition, the use of anti-nucleocapsid antibody tiers may significantly underestimate the prevalence of breakthrough infections in vaccinated individuals.

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Address correspondence to Stavros Garantziotis, garantziotis@niehs.nih.gov.

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It is impossible to overstate the impact of COVID-19 on health care institutions, economies, and societies worldwide. It is also impossible to overstate the tremendous medical and scientific response to COVID-19, which led to the development of novel vaccines and treatments in record time (1, 2). As the SARS-CoV2 virus evolved, so did the medical and public health approaches by which to combat this pandemic. Therefore, it is important to continue research into the natural history and progression of COVID-19 in real-world settings so as to better adapt our prevention and treatment strategies.

Billions of people have received one or more shots of various SARS-CoV2 vaccines (https://ourworldindata.org/covid-vaccinations?country=OWID_WRL, accessed 4/13/2022); however, information on several aspects of the vaccine response is lacking, including a thorough, longitudinal follow-up in antibody titers after infection or vaccination. This study followed 629 subjects in central North Carolina in the United States of America and evaluated antibody titers at 3-month intervals over 1 year. Information was collected on self-reported (but medically diagnosed) COVID-19 incidence and vaccination status. We report several notable findings, including an association of vaccination with more cautious social behavior, an association of antibody titers with breakthrough infections, and the behavior of antibody responses to different vaccines and infections with SARS-CoV2.

RESULTS

Descriptive statistics. 629 subjects were recruited, including 387 women and 226 men (16 not reported) (Table S1). The population was skewed toward Caucasian (85.3%), with 3.8% Black/African-American and 5.7% Asian (17 not reported). 82.8% of the population reported receiving a COVID-19 vaccine, which is somewhat higher than the overall vaccination profile of North Carolina, where approximately 72% of the adult population is vaccinated (https://covid19.ncdhhs.gov/dashboard/vaccinations). The majority of the participants received mRNA vaccines. 90 cases (14.3%) of medically confirmed COVID-19 infection were reported before or during the study. The incidence of infection followed the general trends of COVID-19 infection in North Carolina, with noticeable increases during the Delta and Omicron waves (Fig. S1). We did not perform genotyping of virus variants. However, given that our population was geographically defined in central North Carolina, we relied on the North Carolina Department of Health and Human Services surveillance data (https://covid19.ncdhhs.gov/dashboard) to support the claim that the viral epidemiology in our area followed national trends, with the α- and β-variants predominant in 2020, the δ-variant present in most of 2021, and the o-variants taking over after December 2021. We tested every subject with a reported infection for persistent SARS-CoV-2 carriage via a nasal PCR test. None of the subjects had a positive test after a symptomatic recovery from infection, supporting that SARS-CoV2 infection does not lead to asymptomatic carriage status.

Association of socioeconomic factors and behavior with vaccination and infection. We analyzed the association of socioeconomic factors, behavioral characteristics, and health history (Table S2) with COVID-19 diagnosis, breakthrough COVID-19 infections, and COVID-19 vaccination. A univariate analysis revealed associations between COVID-19 diagnosis and race, known COVID-19 exposure, degree of socialization, number of adult children living in the same household, and having asthma. The two variables remaining in the final logistic regression after stepwise variable selection were known COVID-19 exposure Odds ratio [OR] = 11.2, 95% confidence interval [CI] = 6.5 to 19.1, $P = 1.8 \times 10^{-18}$) and having asthma (OR = 2.1, 95% CI = 1.1 to 3.9, $P = 0.019$). Increased age, taking medicine for high blood pressure, and a history of autoimmune disease, such as arthritis, rheumatoid arthritis, gout, lupus, or fibromyalgia, were associated with an increased risk of a breakthrough COVID-19 infection, while on-site work decreased the risk in the univariate analysis. After adjusting for covariates, a multivariate analysis only identified an association of breakthrough infection with race (self-identifying as Black/African American versus White/Caucasian (OR = 5.5, 95% CI = 1.6 to 18.4, $P = 0.006$), and a marginally significant association with increased age (OR = 1.03, 95% CI = 1 to 1.06, $P = 0.078$).

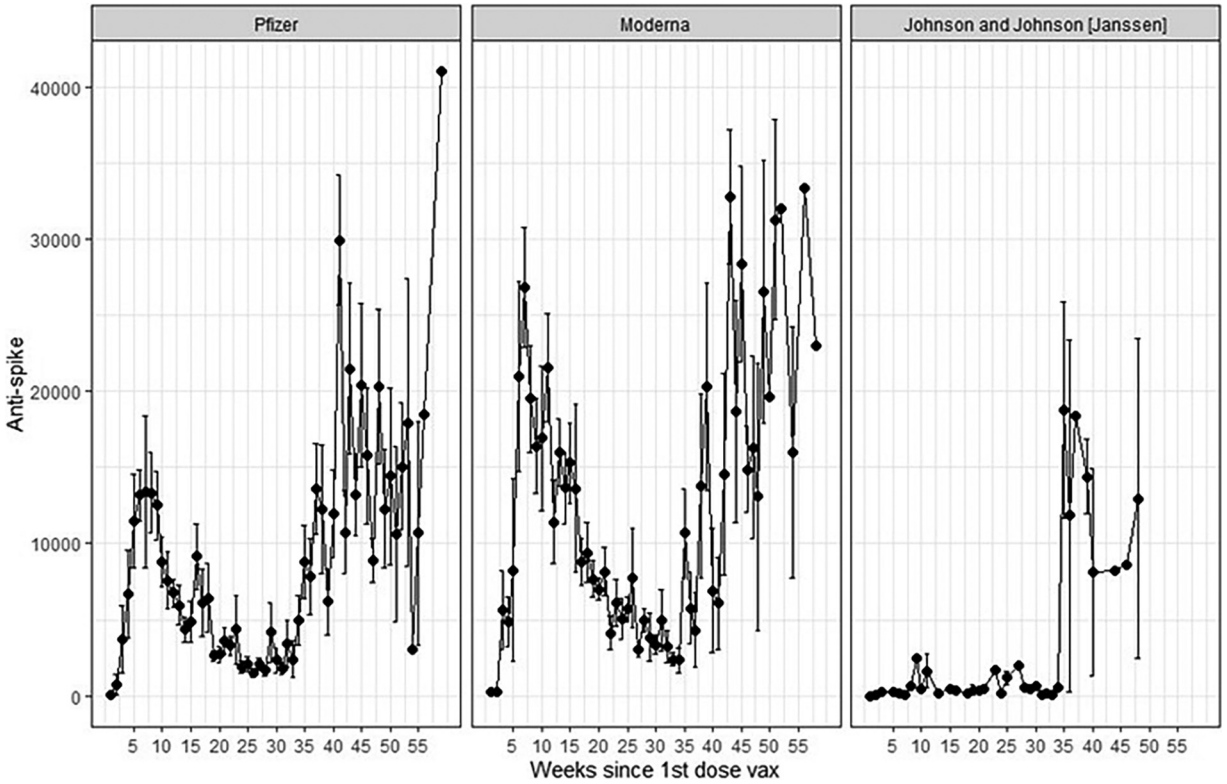

FIG 1 Weekly average of anti-spike levels as a function of time since vaccination for different vaccine manufacturers. The *y* axis denotes the average anti-spike level ± standard error, and the *x* axis denotes the number of weeks since the first dose of COVID-19 vaccination. The increase in titers around week 35 represents participants receiving booster vaccinations, which occurred at a different time point for each participant. Subjects with breakthrough infections were excluded from this analysis. Pfizer: BNT162b2 vaccine (Pfizer-BioNTech); Moderna: mRNA-1273 vaccine (Moderna); Johnson and Johnson (Janssen): Ad26.COV2.S vaccine (Johnson&Johnson/Janssen).

Many factors were either positively or negatively associated with COVID vaccination, including older age, degree of socialization, education, marital status, on-site work, owning a home, having children younger than 18 years of age, size of household, known COVID-19 exposure, having had a flu shot in the past 12 months, and having an autoimmune disease. In the multivariate analysis, known COVID-19 exposure (OR = 0.52, 95% CI = 0.29 to 0.94, $P = 0.31$), a high degree of socialization during the pandemic (OR = 0.64, 95% CI = 0.48 to 0.85, $P = 0.0021$), and having children younger than 18 years of age (OR = 0.7, 95% CI = 0.54 to 0.91, $P = 0.0065$) were negatively associated with vaccination.

Anti-spike antibody response depends on vaccine type. We first described the anti-spike antibody response after vaccination. We observed the expected increase, followed by a gradual waning of the anti-spike antibody titers (Fig. 1; Fig. S2 and S3) and then a subsequent increase after booster vaccination (Fig. 1; Fig. S4). As previously described (3–5), the Moderna vaccine induced a somewhat higher anti-spike antibody titer than did the Pfizer vaccine, and the difference persisted over at least 6 months after the initial vaccination cycle. We developed a linear mixed effects model to predict the response to the anti-spike antibody vaccine over time among the vaccinated individuals. There were significant inter-individual differences in vaccine responses. The most significant association on a population level was with age, as older individuals tended to have lower anti-spike responses; however, this association was no longer significant after adjusting for other covariates. In the multivariate analysis, the Moderna vaccine induced anti-spike levels 2.38 times higher than those induced by the Pfizer vaccine (95% CI: 1.97 to 2.87, $P = 6.2 \times 10^{-18}$). Interestingly, participants who had received the Ad26.COV2.S vaccine had fundamentally different antibody responses; their anti-spike levels remained below 1,000 AU/mL (22% of the Pfizer-induced level, 95% CI: 14% to 33%, $P = 7.4 \times 10^{-12}$) without a significant change being observed throughout the study period (Fig. 1; Fig. S2 to 4). Booster vaccination and

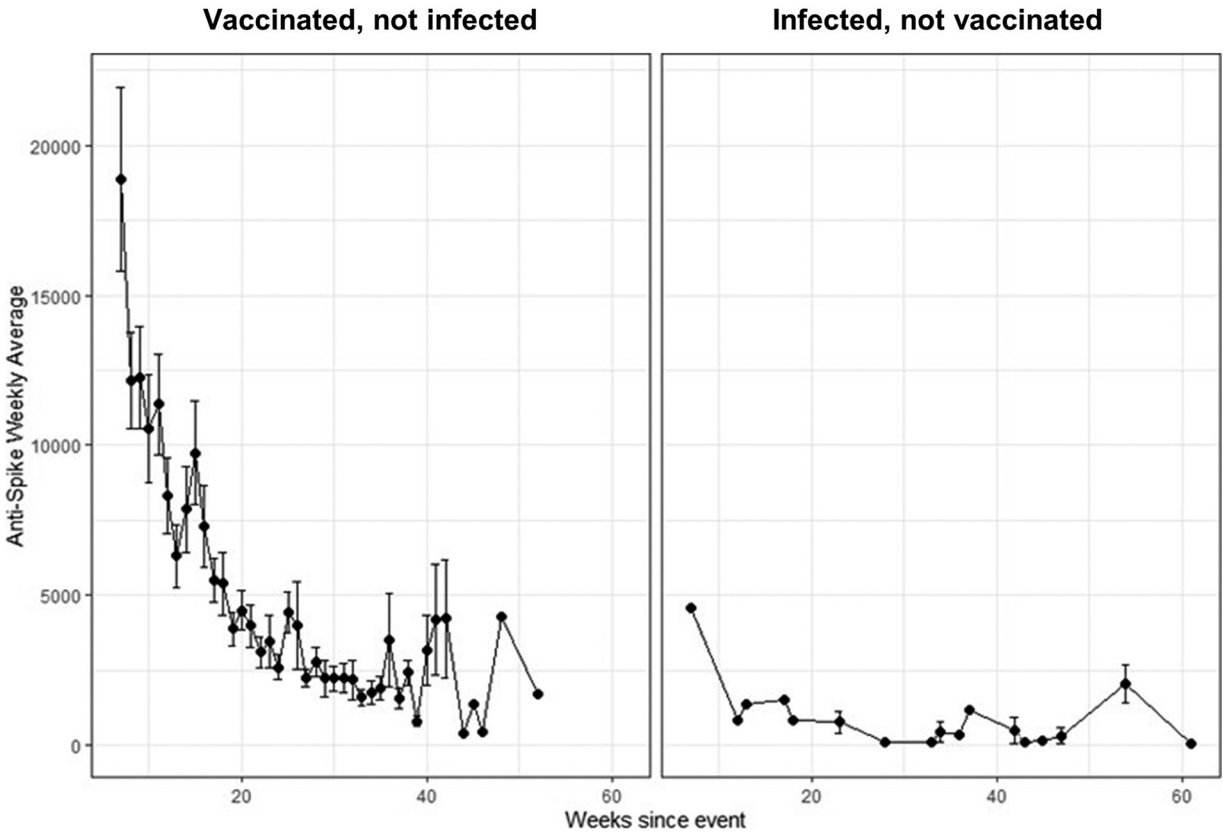

FIG 2 Weekly average of anti-spike levels in the vaccinated but never infected subjects versus the never vaccinated but infected subjects. The *y* axis denotes the average anti-spike level ± standard error, and the *x* axis denotes the number of weeks since COVID-19 diagnosis or vaccination.

previous or subsequent COVID-19 infections led to an increase in anti-spike antibody titers (9.763-fold, 95% CI = 8.06 to 11.81, $P = 3.4 \times 10^{-93}$ for booster vaccination and 3.27-fold, 95% CI = 2.57 to 4.15, $P = 3.59 \times 10^{-21}$ for previous COVID-19 infection) (Fig. 1; Fig. S4). Males had lower anti-spike antibody levels than did females (73%, 95% CI = 62% to 87%, $P = 3.6 \times 10^{-21}$). Significant reductions in anti-spike levels were also observed in those taking immune-suppressing medications (27%, 95% CI = 16% to 46%, $P = 1.68 \times 10^{-6}$) and those with an autoimmune disease (73%, 95% CI = 59% to 90%, $P = 0.00049$) or kidney disease (31%, 95% CI = 15% to 62%, $P = 9.6 \times 10^{-4}$). We compared anti-spike responses in subjects who were infected but never vaccinated versus those who were vaccinated but never infected and had not yet received a booster vaccination (Fig. 2). We observed the induction of much higher anti-spike levels by vaccination than after infection; anti-spike levels stayed above 1,000 AU/mL even 10 months after vaccination, while the levels dropped to below 1,000 AU/mL within 4 months after infection in those who were never vaccinated. The anti-spike levels in those who were infected but never vaccinated were only 20.5% (95% CI = 11.6% to 36.4%, $P = 9.16 \times 10^{-8}$) of those who were vaccinated but never infected.

Breakthrough infections do not generate a reliable anti-nucleocapsid antibody response. We then evaluated the incidence and kinetics of anti-nucleocapsid responses after a reported infection (Fig. 3). In unvaccinated individuals, infection led to a significant increase in anti-nucleocapsid antibodies, which persisted over several weeks, as expected. However, in vaccinated individuals, breakthrough infections led to a much more muted response of only 54% (95% CI = 35% to 83%, $P = 0.0045$) of what was observed in the unvaccinated individuals, with several subjects not seroconverting their anti-nucleocapsid antibody titers after a documented infection.

There is a titer threshold for antibody protection. We evaluated whether there is a threshold of antibody titers associated with breakthrough infections (Fig. 4A). There was a clear association of breakthrough cases with lower anti-spike antibody titers, with 80% of breakthrough infections occurring at titers of <3,000 AU/mL and 90% at titers of <3,600 AU/

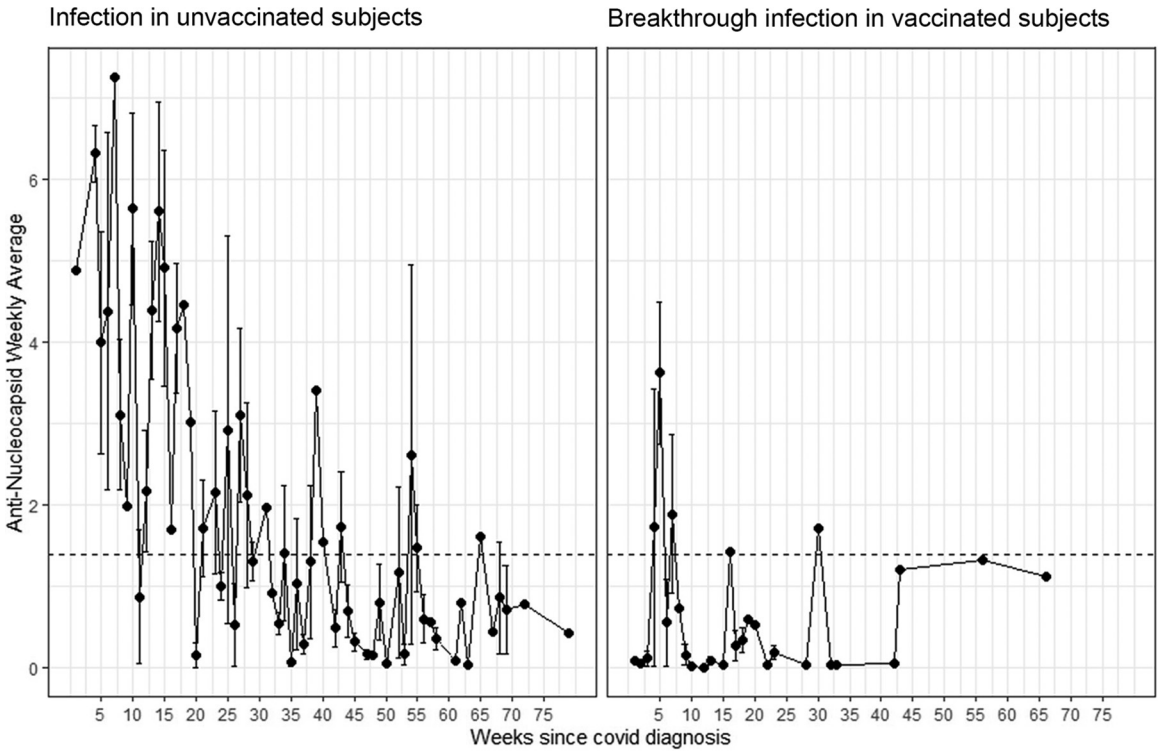

FIG 3 Weekly average of anti-nucleocapsid levels by breakthrough infection status. The *y* axis denotes the average anti-nucleocapsid level ± standard error. The *x* axis denotes the number of weeks since COVID-19 diagnosis. The dotted line denotes the threshold for a positive result per the manufacturer's instructions.

mL. This association is also demonstrated by the decreasing trend in the proportion of breakthrough cases as the anti-spike levels increased (Fig. 4B and C; Fig. S5). However, there was a significant overlap of anti-spike antibody titers between the infected and uninfected participants (Fig. 4C).

To assess the neutralization ability of anti-spike antibodies against different COVID-19 variants, we performed *in vitro* neutralization assays using sera with defined anti-spike antibody titers (Fig. 5). There was inhibition of the Delta variant infection by sera with titers >2,000 AU/mL but not by sera with titers below this level. As previously published (6–8), we confirmed that the Omicron variant evaded antibody neutralization to a significant degree compared to the Delta variant (Fig. 5).

DISCUSSION

SARS-CoV2 and COVID-19, along with the medical and societal responses to it, continue to evolve as new virus variants, vaccine strategies, and treatment approaches appear. This study addressed several points of interest with regard to vaccination behavior, vaccination responses, and protection against infection with SARS-CoV2. Several novel observations are noteworthy: the association of vaccination status with socialization behavior, qualitatively different anti-spike responses depending on vaccine type, the absence of a robust anti-nucleocapsid response in breakthrough infections, and the identification of an anti-spike antibody titer that is associated with breakthrough infection.

To our knowledge, this is the first study to evaluate the social behavior of participants in conjunction with vaccination status. Perceptions of COVID-19 risk play a role in seeking vaccination and avoidance strategies, such as masking and limiting social interactions (9). Although some studies suggested that concerns about safety were most relevant in the decision to forgo SARS-CoV-2 vaccination (10, 11), our study clearly suggests a strong negative association between not being vaccinated and taking fewer precautions in the social interactions of our study participants. Thus, individuals who did not perceive COVID-19 as a significant threat to their health may have resisted changes to their behavior in response to the pandemic, in

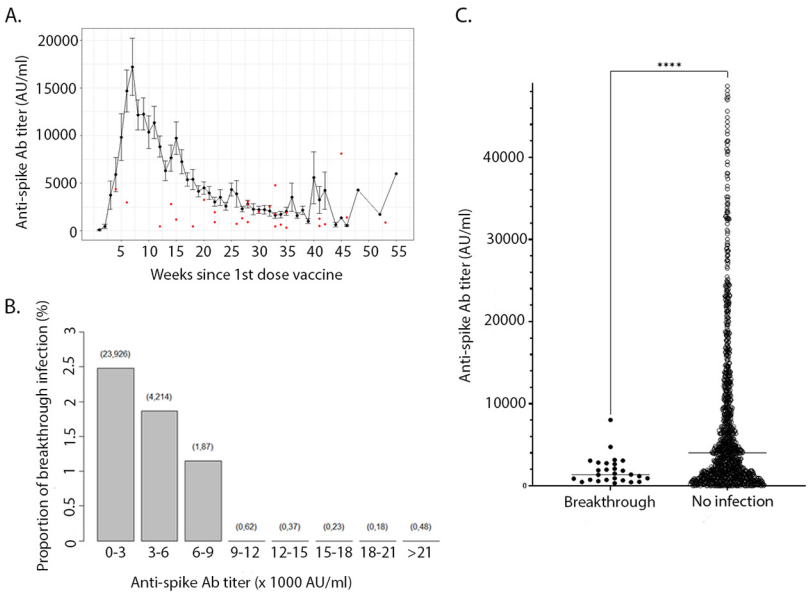

FIG 4 Anti-spike titers in relation to breakthrough infections. (A) Anti-spike titers (weekly mean ± standard deviation) in the vaccinated but not infected subjects versus the predicted titers (red dots) at the time of infection for breakthrough infections. The *y* axis denotes the anti-spike levels ± standard error (AU/mL) among the subjects who were vaccinated but not infected. The *x* axis denotes the number of weeks since the first dose of COVID-19 vaccination. Predicted titers for breakthrough infection are plotted at each time point of diagnosis. (B) Proportion of breakthrough cases in relation to the total number of participants in the same bin of antibody titers, binned in 3,000 AU/mL titer increments. The numbers above each bar show the number of breakthrough cases and the total number of measurements at each level, separated by a comma. (C) Antibody titers are significantly lower in patients with breakthrough infections than in uninfected patients. ****, $P < 0.0001$.

terms of both social interactions and vaccination. This conclusion is strengthened by our finding of a negative association between exposure to SARS-CoV2 and vaccination, suggesting a higher exposure potential among the unvaccinated participants. Since there was also the expected positive association of infection and known exposure to COVID-19, another conclusion from our study is that in real-life situations, the protective effect of vaccination in preventing COVID-19 may conflate vaccine-mediated protection with protection obtained via the more cautious behavior displayed by vaccinated individuals. Importantly, we evaluated all participants with a reported infection for the persistence of SARS-CoV2 in the upper airways via a polymerase chain reaction (PCR) assay. None of our recovered participants had detectable virus, suggesting that there is no human asymptomatic carrier depot for SARS-CoV-2 after recovery, an encouraging finding. We also noted a positive association between prior influenza vaccination and current COVID-19 vaccination. This confirms previous studies (9) and may reflect a positive attitude toward vaccination in general or medical conditions that move toward seeking vaccination.

We found a pronounced difference in anti-spike antibody titers between the mRNA vaccines and the adenoviral vaccine Ad26.COV2.S, confirming previous findings (12, 13). This may help explain the somewhat reduced performance of Ad26.COV2.S in the protection against SARS-CoV2 in clinical trials (14). It is also notable that, unlike those of the mRNA vaccines, the Ad26.COV2.S antibody response did not wane over time in our cohort, again confirming previous reports (13, 15). Despite differences in antibody responses, both types of vaccines induce similar and durable cellular immunity responses (13, 15–17). Thus, in aggregate, our results support a qualitatively different anti-spike antibody response to the two studied mRNA vaccines compared to those of the adenovirus-based vaccine Ad26.COV2.S. This leads to substantially different circulating antibody levels. Thus, in terms of evaluating the immune response, anti-spike antibodies may have especially limited information value in patients receiving the adenoviral vaccine.

Another notable finding from our study was that breakthrough infections often did

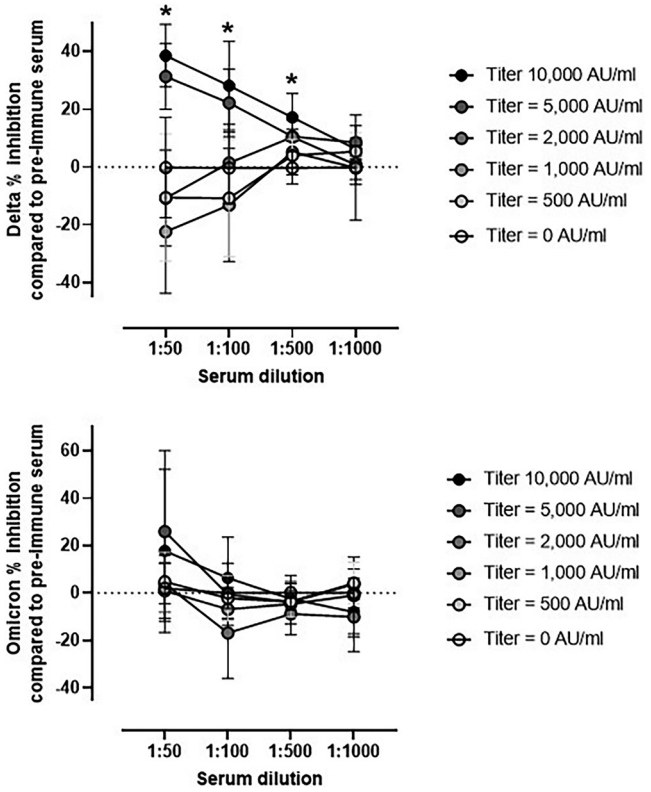

FIG 5 Neutralization capacity of immune sera with specified anti-spike antibody titers against pseudovirions expressing the Delta (top panel) or Omicron variant (bottom panel) spike proteins, expressed as % inhibition above the nonspecific preimmune serum ± standard error (i.e., sera collected prior to 2019) *, $P < 0.05$, ANOVA with the Sidak correction for multiple comparisons.

not generate a robust anti-nucleocapsid antibody response, which, to our knowledge, has not been reported previously. This may be because the already present immunity leads to a sufficiently effective response so as to render the further development of antibodies unnecessary. It is also important to note that we only utilized a single assay. As such, the results might have differed if other assays had been used. However, this assay has performed well in comparative analyses, particularly in the early time points postinfection (18). Thus, our findings support that breakthrough infections may induce a different immune response than do infections in naive individuals (i.e., a focused boosting of anti-spike immunity as opposed to a broad induction of immunity against other SARS-CoV-2 epitopes) and suggest that evaluating the prevalence of infection via anti-nucleocapsid serology may significantly underestimate the true prevalence of breakthrough infections in previously vaccinated populations. We also found that natural immunity without vaccination led to a significantly lower and shorter-lived anti-spike response compared to vaccination. It should be noted that our participants reported mild to moderate infection severity. Disease severity is associated with the robustness of the immune response (19, 20). Thus, our data support that an infection of mild or moderate severity is inferior to vaccination in the generation of anti-spike antibody titers.

Currently, recommendations for booster shots are based solely on chronological and demographic determinants (e.g., time from previous immunization, age, comorbid conditions, etc.). We questioned whether antibody titers can be utilized to individualize this decision and predict a clinically relevant threshold level which indicates an elevated risk of a breakthrough infection. Our results suggest that antibody levels may have some utility as decision aids for booster vaccination. There was a clear association of anti-spike antibody titers with breakthrough infections such that 80% of breakthrough infections occurred in participants with anti-spike titers of $<$3,000 AU/mL. Thus, this study supports that there may be added value in utilizing antibody titers as a positive predictor of the need for a booster if

levels are below a given threshold. However, there was a significant overlap between the antibody levels in participants with and without a breakthrough infection. Thus, antibody levels cannot be the only or the major deciding factor in a clinical setting. Also, as mentioned above, participants receiving adenoviral vector vaccines, such as the Ad26.COV2.S, have qualitatively different antibody responses and therefore cannot utilize antibody titers in their decision process.

Using an *in vitro* neutralization assay, we also addressed the question of whether antibody titers are representative of intrinsic protective capacity or whether they are simply biomarkers of immune protection. In the former case, we might expect the *in vitro* protective titer threshold to be similar to the *in vivo* clinical protection threshold. Indeed, we found that titers above 2,000 AU/mL inhibited infections with the Delta variant, while titers at or below this threshold had no inhibitory activity. This is similar to the threshold of 3,000 AU/mL, below which breakthrough infections were more common. Notably, and in agreement with other reports on immune evasion by the Omicron variant (8, 21, 22), there was no *in vitro* inhibitory activity of immune sera against the Omicron variant. This suggests that any antibody titer criteria for boosters would have to be reevaluated for each novel variant, further limiting the clinical utility of antibody titers as a decision-making tool.

There are several weaknesses to this study. We followed antibody levels quarterly from study enrollment, and the follow-ups were not fixed to vaccination or to any other clinical event. Thus, there is some degree of missing information in our data. Also, we measured only antibody responses and did not address the question of cellular immunity in response to infection, vaccination, or booster shots or in the protection against COVID-19. At this point, there are no broadly applicable, clinically approved tests for the evaluation of cellular immunity to SARS-CoV2. Thus, we limited our evaluation to tests that may have the potential for clinical application and medical decision-making. There is no doubt that high-throughput cellular immunity assays would have great utility in further evaluating the effects of vaccines and in medical decision-making regarding the need for vaccination or boosters. Also, even though we followed several hundred participants, our study had a relatively limited sample size for the detection of COVID-19 infections. As such, the results can be viewed as hypothesis-generating and are not definitive. However, several findings from our study population replicate those of previous studies, increasing our confidence in the validity of our results.

In conclusion, our study identified novel associations of anti-spike and anti-nucleocapsid antibodies with breakthrough COVID-19 infections. Our results may aid in the refinement of vaccination strategies during this ongoing pandemic.

MATERIALS AND METHODS

Assay information. Whole blood was collected, processed into serum, and immediately stored at −80°C until tested. We used clinically validated assays on an Architect i1000sr analyzer (Abbott, Chicago, IL). Anti-spike antibodies were quantitatively analyzed using an AdviseDx SARS-CoV-2 IgG II assay (CMIA, Abbott). The assay detects IgG antibodies specific to the receptor binding domain (RBD) of the S1 subunit of the spike protein of SARS-CoV-2. The quantitative analytical extended measuring interval was 22.0 to 50,000.0 AU/mL. Anti-nucleocapsid IgG antibodies were analyzed using a SARS-CoV-2 IgG assay (Abbott), and results were generated as a signal-to-calibrator (S/C) index. In accordance with the manufacturer's instructions, an anti-N Index was considered positive at a value of ≥1.4. All manufacturer performance characteristics were verified in accordance with the CLIA laboratory requirements for *in vitro* diagnostic testing.

Viral detection. Mid-turbinate nasal swabs were collected from participants who reported either a COVID-19 diagnosis by a medical professional or a positive result by a rapid test as well as from participants who had new positive anti-nucleocapsid antibodies. These were frozen in viral transport media and transported by courier to a commercial testing facility for real-time reverse transcription PCR.

Study subject enrollment and data collection. The study received approval from the NIH Institutional Review Board and was entered in the clinicaltrials.gov website (NCT04490174). Recruitment began in August 2020 and continued until April 2022. Participants were recruited from the general population using print materials, newsletters, electronic materials, newspaper advertisements, a study-specific website, and social media postings. Study data were collected and managed using the REDCap electronic data capture tools hosted at the NIEHS. The data output used for the analyses were generated using SAS 9.4 (SAS Institute, Inc., Cary, North Carolina).

***In vitro* SARS-CoV2 pseudovirion neutralization assay.** SARS-CoV2 spike pseudotyped lentiviruses were produced in HEK293T/17 cells (ATCC number CRL-11268) according to published methods (23). Pelleted virus was resuspended in PBS and stored at −80°C.

24 h prior to the neutralization assays, HEK293Ts expressing human angiotensin-converting enzyme 2 (HEK293T-ACE2, BEI Resources, NR-52511) were plated at the density of 50,000 cells/well in 24-well plates. Patient sera were diluted 1:50, 100, 500, and 1,000, and these were used to treat SARS-CoV-2 spike (Delta or Omicron) pseudotyped recombinant lentiviruses expressing RFP (S-CD512-EF1a-RFP). The pseudotyped viruses were incubated with patient sera for 1 h at 37°C in transduction media (DMEM + 2% FBS). After incubation, the cell medium was replaced with serum-treated virus, media alone (negative-control), or untreated virus (positive-control). 24 h later, medium was replaced with growth media (DMEM + 10% FBS). 48 h postransduction, the cells were harvested and fixed in 1% formaldehyde. A BD LSRFortessa Flow Cytometer was used to determine the percentage of fluorescent cells. All experiments were performed in duplicate.

Statistical analysis. The statistical analyses were performed using R (version 4.1.3). In a univariate analysis, a Student's *t* test was used to compare continuous variables, and Fisher's exact test was used to compare categorical variables. We used multivariate logistic regression to evaluate the associations between COVID-19 diagnosis, breakthrough infection, and COVID-19 vaccination. Variables with a *P*-value of <0.1 in the univariate analysis were considered covariates in the multivariate logistic regression analysis, and stepwise selection was used to identify the final models. Due to the skewed distribution of the anti-spike antibody levels, we log-transformed the values before analysis and fit the data using a linear mixed model with random intercepts for each subject to account for the correlation between multiple measurements from the same subject. The predictors in the linear mixed model include natural cubic splines of the time (days) between vaccination and anti-spike measurement (to account for the changes of anti-spike antibody titers over time) and other covariates, as determined by the stepwise regression analysis. For the anti-nucleocapsid analysis, we treated S/C levels of <0.5 as zero and conducted the analysis using a model for the zero-inflated gamma distribution with repeated measures, as implemented in the R package glmmTMB. The predictors in the anti-nucleocapsid model include natural cubic splines of the time (days) between COVID-19 infection and anti-nucleocapsid measurement (to account for the changes of anti-nucleocapsid antibody titers over time) and other covariates, as determined by the stepwise regression analysis.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENT

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