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Immunity to infection

Short Communication

Neutralizing antibody responses 300 days after SARS-CoV-2 infection and induction of high antibody titers after vaccination

Doris Urlaub[1], Natalie Wolfsdorff[1], Jan-Erik Hoffmann[2], Stefanie Dorok[2], Markus Hoffmann[3,4], Moritz Anft[5], Naomi Pieris[1], Patrick Günther[6], Bernhard Schaaf[7,8], Uwe Cassens[9], Peter Bröde[1], Maren Claus[1], Lea K. Picard[1], Sabine Wingert[1], Simone Backes[10], Deniz Durak[11], Nina Babel[5], Stefan Pöhlmann[3,4], Frank Renken[11], Stefan Raunser[6] and Carsten Watzl[1]

[1] Department for Immunology, Leibniz Research Centre for Working Environment and Human Factors (IfADo) at TU Dortmund, Dortmund, Germany
[2] Protein Chemistry Facility, Max Planck Institute of Molecular Physiology, Dortmund, Germany
[3] Infection Biology Unit, German Primate Center - Leibniz Institute for Primate Research, Göttingen, Germany
[4] Faculty of Biology and Psychology, Georg-August-University Göttingen, Göttingen, Germany
[5] Center for Translational Medicine and Immune Diagnostics Laboratory, Marien Hospital HerneUniversity Hospital of the Ruhr-University Bochum, Herne, Germany
[6] Department of Structural Biochemistry, Max Planck Institute of Molecular Physiology, Dortmund, Germany
[7] Department of Respiratory Medicine and Infectious Diseases, Klinikum Dortmund, Dortmund, Germany
[8] Faculty of Health, University Witten/Herdecke, Herdecke, Germany
[9] Institute for Transfusion Medicine, Laboratory Medicine and Medical Microbiology, Medical Center Dortmund, Dortmund, Germany
[10] Institute for Virology and Immunobiology, University of Wuerzburg, Wuerzburg, Germany
[11] Dortmund Health Department, Dortmund, Germany

Neutralizing antibodies against SARS-CoV-2 are important to protect against infection and/or disease. Using an assay to detect antibodies directed against the receptor binding domain (RBD) of SARS-CoV-2 Spike, we identified individuals with SARS-CoV-2 infection after an outbreak at a local health institution. All but one COVID-19 patient developed detectable anti-RBD antibodies and 77% had virus neutralizing antibody titers of >1:25. Antibody levels declined slightly over time. However, we still detected virus neutralizing antibody titers in 64% of the COVID-19 patients at >300 days after infection, demonstrating durability of neutralizing antibody levels after infection. Importantly, full COVID-19 vaccination of these individuals resulted in higher antibody titers compared to fully vaccinated individuals in the absence of prior infection. These data demonstrate long-lived antibody-mediated immunity after SARS-CoV-2 infection, and a clear benefit of two vaccine doses for recovered individuals.

Keywords: SARS-CoV-2 · COVID-19 · Vaccines · antibody titers · waning immunity

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Correspondence: Professor Carsten Watzl
e-mail: watzl@ifado.de

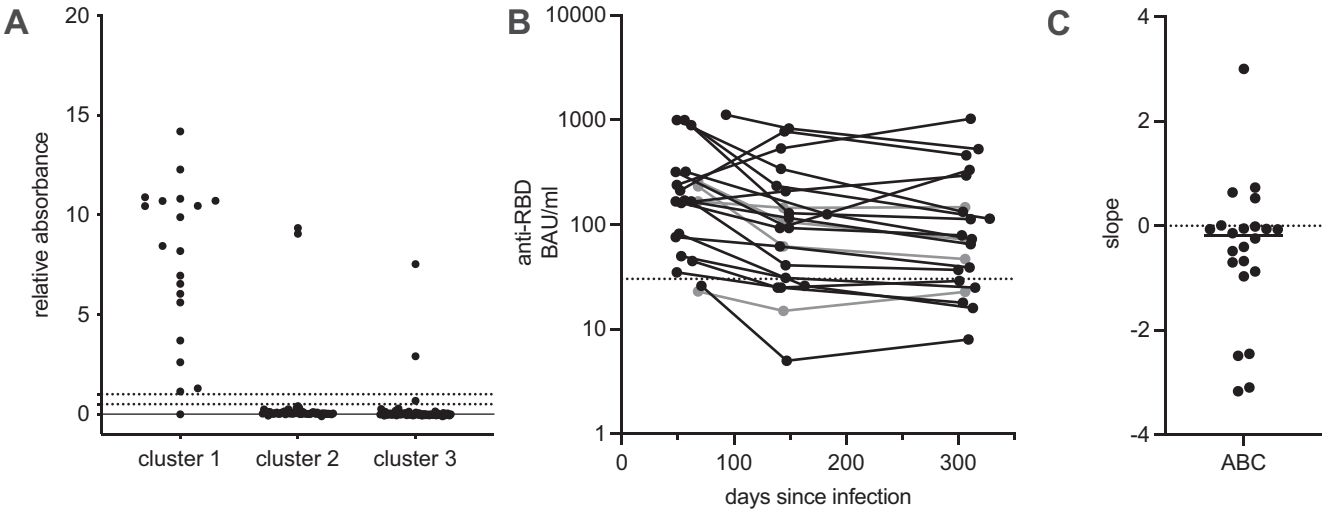

Figure 1. Detecting and examining the durability of anti-RBD antibody levels. (A) ELISA to detect SARS-CoV-2-specific antibodies using the RBD of the spike protein as antigen. Dotted lines indicate the thresholds for positive (>1) and negative (<0.5) signals. Twenty people from cluster 1 were tested positive by PCR, 55 people in cluster 2 had known interactions with cluster 1, 73 people in cluster 3 were not knowingly in contact with infected people. Dots are single values combined from a total of four assays. (B) Twenty-two of the individuals who were tested positive for antibodies during the first round of sampling (sample A) were tested again at about 150 days (sample B) and 300 days (sample C) after the infection. The anti-RBD antibody level calibrated to the WHO standard is displayed over time, the dotted line at 30.3 BAU/mL indicates the threshold for positive values on this scale. Eighteen PCR positive people from cluster 1 are shown in black (the date of the positive PCR test was set as timepoint 0) and four people without PCR confirmed infection in are shown in grey (mean timepoint of the outbreak was set to 0). (C) A simple linear regression was used to calculate the slopes that indicate the changes of signal intensity between the timepoints A to C. Changes are shown as individual values and the median as horizontal bar. (A) Measurements for cluster 1 and positive samples from cluster 2+3 are representative of three independent experiments. (B-C) Antibody titers were determined by measuring four different dilutions of each sample.

Introduction

Most people develop antibodies against SARS-CoV-2 during infection, which not only provide immunity to reinfection but can be used to identify previously infected individuals [1]. For virus neutralization, antibodies directed against the SARS-CoV-2 spike protein and especially antibodies against the receptor binding domain (RBD) are important [2]. Therefore, most COVID-19 vaccines use the spike protein as an antigen and there is a good correlation between vaccine efficacy and the levels of neutralizing antibodies [3, 4], suggesting that antibody titers are important for immunity against SARS-CoV-2. Recently, the waning of antibody titers within 6 months after full vaccination has gained much attention, resulting in a reduced protection from symptomatic infection [5–9]. Therefore, booster vaccinations have been recommended in many countries. However, longitudinal data about the durability of antibody titers after SARS-CoV-2 infection and the effects of full vaccination of recovered individuals are scarce.

Here, we use an ELISA to detect anti-RBD-specific antibodies [10]. We used this assay to detect previously infected individuals in a local outbreak and to follow the stability of anti-RBD antibodies and their virus neutralizing activity up to 300 days after infection. Finally, our data show that full vaccination of recovered individuals at >300 days after SARS-CoV-2 infection results in higher antibody titers compared to fully vaccinated individuals without prior infection.

Results and discussion

The presence of SARS-CoV-2-spike-specific antibodies is a clear indication of a prior infection in the absence of vaccination. Therefore, we used an ELISA with the RBD of the spike protein of SARS-CoV-2 as an antigen [10], to test volunteers in a public health institution with several documented cases of SARS-CoV-2 infection early during the pandemic (March 2020). We defined three clusters: People in cluster one (n = 20) did have a PCR-confirmed SARS-CoV-2 infection (Supporting information Table S1) 48–93 days before sampling (mean 59 days). People in cluster two (n = 55) did not test positive by PCR but did have contact with individuals from cluster one. Finally, cluster three (n = 73) defined people without positive PCR and without contact to individuals in cluster one. All but one individual from cluster one tested positive for anti-RBD antibodies (Fig. 1A) and we found two individuals in each of the other clusters who had clearly detectable antibodies, strongly suggesting a prior infection that was not diagnosed by PCR. We tested the PCR-positive sample that gave a negative test result in our antibody assay with two different commercial anti-SARS-CoV-2 ELISA and also obtained a negative result. However, we could detect SARS-CoV-2-specific T-cell responses in a blood sample from this individual (data not shown), suggesting that he/she was indeed infected with SARS-CoV-2, but failed to produce detectable anti-RBD antibody levels. This is in line with other reports showing that a small percentage of SARS-CoV-2 infected individuals do not produce detectable antibodies [1]. These data

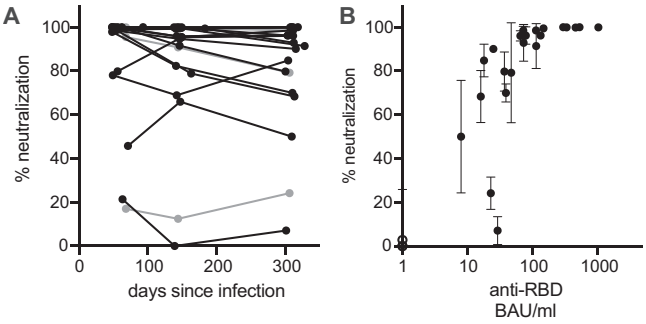

Figure 2. Investigating virus neutralizing antibodies with SARS-CoV-2-spike pseudotyped VSV. (A) The sera of 22 individuals who were tested positive for antibodies by ELISA were used in a neutralization assay against SARS-CoV-2-spike pseudotyped VSV at a dilution of 1:25. Percent neutralization was calculated by setting the value without serum to 0% and a sample without virus to 100% neutralization. Mean values of three independent experiments per sample are shown. (B) Antibody titers of the anti-RBD ELISA versus virus neutralization are shown. Mean and SD from three replicates of the neutralization assay for the timepoint 300 days after the infection is shown as black circles. Three negative controls are shown as open circles. Since anti-RBD values in BAU/mL for these samples could not be determined, it was set to 1 for visualization.

suggest that 15.5% of the individuals in this public health institution were infected with SARS-CoV-2 which was well above the infection rate in the general population at this time.

We had the chance to collect serum samples from 22 of the antibody-positive individuals at about 150 days (138–183) and 300 days (300–328) post infection to determine the durability of anti-RBD antibody levels (Fig. 1B). While we observed a reduction in the ELISA signal (geometric mean titer [GMT] 170 binding antibody units [BAU]/mL [95% confidence of interval [CI]: 102, 284] at 50 days, GMT 90 BAU/mL [95% CI: 50, 159] at 150 days, and GMT 78 BAU/mL [95% CI: 45, 136] at 300 days), anti-RBD antibodies were still clearly detectable in 73% (16 of 22) of the individuals >300 days post infection. This demonstrates that anti-SARS-CoV-2-specific antibodies are stable for at least 300 days post infection. The rate of reduction of the ELISA signal was quite stable over time (Fig. 1C), and comparable to previous reports [1, 11–13], but antibodies levels declined slower compared to antibody titers after immunization [14].

For immunity after SARS-CoV-2 infection, virus neutralizing antibodies play an important role. We, therefore, tested the serum samples for their activity in neutralizing infections of SARS-CoV-2-spike pseudotyped VSV as previously described [15]. When testing the samples 59 days after infection, we found full virus neutralization (>90%) at 1:25 serum dilution in 77% (17 of 22) of the individuals (Fig. 2A). Also, virus neutralization activity declined over time and in samples from 300 days post infection we found >90% virus neutralization in 64% (14 of 22) of the individuals. When we compared anti-RBD antibody levels to the virus neutralization activity 300 days after infection, we observed a clear correlation. Anti-RBD antibody titers of >100 BAU/mL correlated with >90% virus neutralization activity at 1:25 serum dilution (Fig. 2B). However, anti-RBD antibody titers of below 100 BAU/mL did not show full virus neutralization, and the extent of virus neutralization was quite variable between individuals. This demonstrates that not all anti-RBD antibodies have neutralizing activity, but above a threshold of 100 BAU/mL our ELISA result could reliably predict virus neutralization. Neutralization assays were performed with the pseudotyped viruses carrying the spike protein of the original Wuhan strain of SARS-CoV-2, as the infections occurred before the emergence of variants of concern. While the data show a relative stability of neutralizing antibodies, neutralization of variants of concern, especially Omicron, would be much reduced.

As recommended, most of the previously infected individuals got vaccinated >300 days after their infection using different COVID-19 vaccines. In all cases, the vaccination resulted in a strong increase in anti-RBD titers (Fig. 3A–C), even in the individual who failed to develop detectable antibodies after the infection (Fig. 3C). However, the titers differed depending on the vaccines used. Vaccination with AZD1222 resulted in a GMT of 895 BAU/mL (95% CI: 362–2215), heterologous vaccination with AZD1222 followed by BNT162b resulted in GMT 3929 BAU/mL (95% CI: 2235–6906), and vaccination with BNT162b2 resulted in GMT 4581 BAU/mL (95% CI 1999–10496). As most previously infected individuals received two vaccine doses, we were interested to compare the anti-RBD titers to fully vaccinated individuals without prior infection. Full vaccination of individuals without prior infection with AZD1222 resulted in a GMT of 238 BAU/mL (95% CI: 185–305), heterologous vaccination with AZD1222 followed by BNT162b resulted in GMT 1180 BAU/mL (95% CI: 972–1432), and vaccination with BNT162b2 resulted in GMT 1151 BAU/mL (95% CI: 828–1600). Therefore, antibody titers were significantly higher in previously infected individuals after full vaccination compared to fully vaccinated individuals without any documented prior infection (Fig. 3D–F). While it may not be surprising that three antigen exposures induce stronger antibody responses [10], this contrasts with other studies that showed that a second vaccination of previously infected individuals did not result in higher antibody titers [16]. However, the long time (>300 days) between infection and vaccination may have contributed to higher antibody titers in our cohort [17].

Concluding remarks

Comparing the antibody titers after infection (Fig. 1) or vaccination (Fig. 3) clearly shows that COVID-19 vaccines can induce higher antibody titers compared to recovered individuals. As our ELISA shows a high correlation with neutralizing antibodies, this may suggest a higher protection from vaccination compared to infection. However, the decline of these antibody titers appears to be faster after vaccination [14], suggesting that recovered individuals retain their immunity for a longer period of time, which is in line with a recent report [18]. More importantly, immunizing recovered individuals with two doses of a COVID-19 vaccine about 300 days after the infection induces significantly higher antibody titers compared to vaccinated noninfected individuals. A recent report suggested that the decline of antibodies in

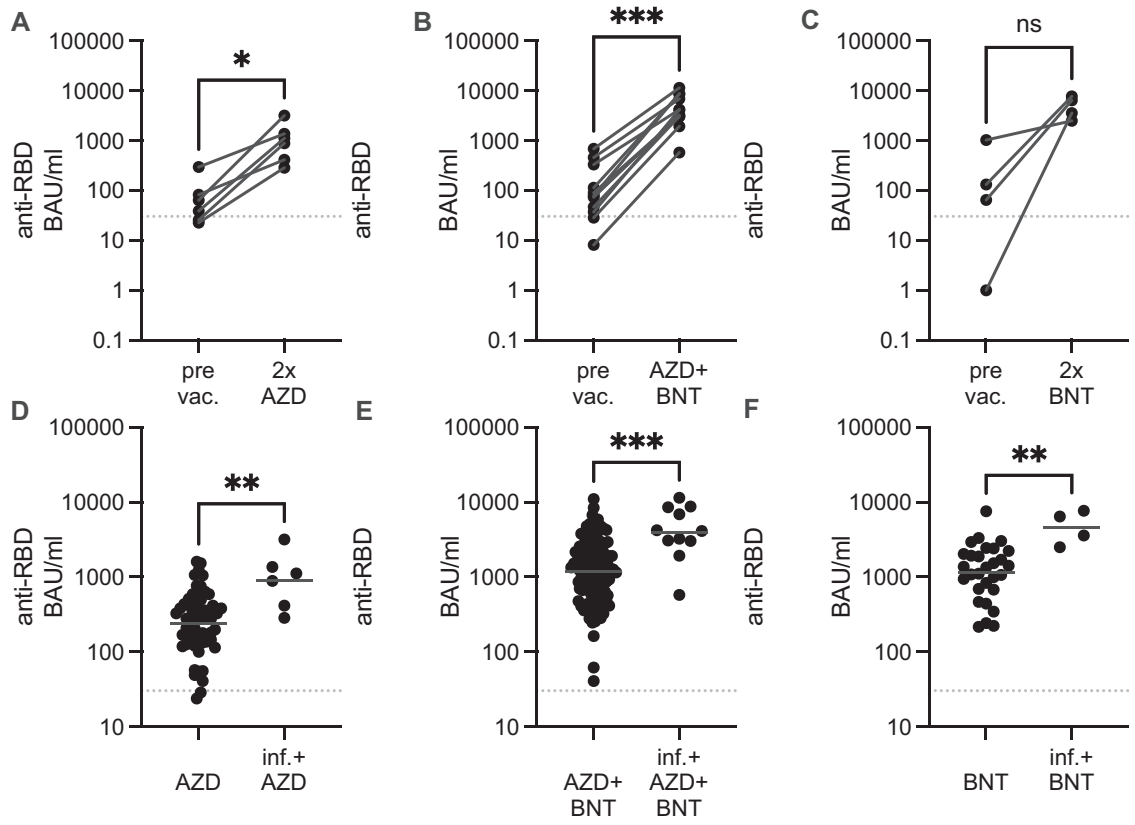

Figure 3. Comparison of titers after SARS-CoV-2 infection and/or vaccination. Previously infected individuals are shown before and after vaccination with two doses of the indicated vaccine combination (A, B, C). Comparison of titers after infection and vaccination versus vaccination only (D, E, F). Shown are values from 6 (A, D), 11 (B, E), 4 (C, F) infected individuals and 59 (D), 106 (E), and 29 (F) uninfected individuals after two doses of the indicated vaccines. Groups were compared using Wilcoxon test (A, B, C) or Mann-Whitney test (D, E, F). Horizontal lines in D, E, and F indicate the geometric mean. (A-F) Antibody titers were determined by measuring four different dilutions of each sample.

vaccinated recovered individuals continues to be slower compared to vaccinated noninfected individuals [17], which would translate in a lasting immunity that could be stable for several years.

Materials and methods

Cell lines

Cells were incubated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (Hek 293T) or 5% (Vero) fetal bovine serum and penicillin/streptomycin (all from Gibco) at 37°C and 5% CO_2 in a humidified incubator and subcultured three times per week.

Serum samples

Samples were taken from venous blood or capillary blood from the fingertip into appropriate tubes (Monovettes or Microvettes with serum gel, Sarstedt). Serum was frozen at −80°C until use.

ELISA

The RBD sequence (spike glycoprotein amino acids 319–541) of the Wuhan strain of SARS-CoV-2 with a C-terminal HIS-tag was expressed in HEK 293-F cells and purified on a HisTrap Excel column using an ÄKTAxpress purification system. Ninety-six well flat bottom plates (maxisorp; Nunc) were coated with 3 μg/mL of SARS-CoV-2 spike RBD overnight at 4°C. Plates were washed, blocked with Biolegend ELISA diluent, and then incubated with serum samples diluted in blocking buffer. The S Antibody (humanized anti-Spike antibody by Dianova/Cusabio, Stock concentration: 0.3 mg/mL) was used as positive control (1:5000 final dilution) and calibrator (1:40 000 final dilution). As a secondary antibody, HRP conjugated anti-human IgG (Dianova), was used and signals were detected with 1 Step Ultra TMB (Pierce). The relative absorbance was calculated using the formula: (sample-negative control)/(calibrator-negative control). Values >1 are positive, values <1 but >0.5 are borderline and should be repeated, and values <0.5 are negative at a 1:100 dilution of the sample. To calculate antibody titers in BAU/mL, the samples were diluted from 1:100 to 1:12 500 and the dilution was calculated for which the relative absorbance would be 1. To do so, hyperbolic curves with the formula $Y = B\max^{*}X/(Kd + X)$ were

fitted using Graph Pad Prism (version 9). Simultaneously, the WHO Standard 20/136, which is defined to have 1000 BAU/mL was also measured to calculate a correction factor to express the dilution values as BAU/mL (Supporting information Fig. S1).

Neutralization assay

VSV*Δ*G*-fLuc were pseudotyped with SARS-CoV-2 spike using either the full-length Spike or a truncated spike (Δ21AA C terminal) according to published methods [15, 19, 20]. Hek 293T cells were transfected with the pCG1-SARS-2-S or pCG1-SARS-2-S-trunc vector using Lipofectamine 2000 (Thermo Fisher Scientific). The next day, these cells were inoculated with a replication-deficient VSV*Δ*G*-fLuc that contains expression cassettes for eGFP and firefly luciferase instead of the VSV-G open reading frame (kindly provided by Gert Zimmer, Institute of Virology and Immunology, Mittelhäusern, Switzerland). After 1 h at 37°C, the cells were washed and fresh medium containing an anti-VSV-G antibody (I1, mouse hybridoma supernatant from CRL-2700; ATCC) was added to neutralize the VSV*Δ*G*-fLuc input virus. SARS-CoV2 spike pseudotyped virus particles were harvested the next day, clarified by centrifugation, and frozen at −80°C until use.

Vero cells were seeded in 96-well plates with white walls and clear bottom to be about 70% confluent the next day. First, the pseudotyped virus with full length or truncated spike was preincubated for 30 min at 37°C with serum diluted at 1:25. Then medium was removed from Vero cells and 40 μL of pretreated virus was added per well. After 1 h at 37°C, 60 μL fresh medium was added and cells were incubated overnight. Neutralization was quantified by measuring firefly luciferase activity using a commercial substrate (Beetle-Juice, PJK), signal intensity from a sample with virus but without serum was interpreted as 0% and signal without virus as 100% neutralization.

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Conflict of interest: The authors declare no conflict of interest.

Ethics approval statement for human studies: This study was approved by the ethics committee of IfADo (#178) and all participants gave informed consent.

Author contributions: D.U., N.W., N.P., M.C., L.P., S.W. planned and caried out the experiments; D.U., P.B., C.W. analyzed the data; M.A., U.C., N.B. provided T cell and additional antibody data; J.-E.H., S.D., P.G. produced and purified recombinant RBD; M.H., B.S., S.B., D.D, S.P, F.R. provided reagents and expertise; S.R. and C.W. supervised the project; and D.U., C.W. wrote the manuscript.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Abbreviations: **BAU**: binding antibody units · **CI**: confidence interval · **GMT**: geometric mean titer · **RBD**: receptor binding domain

Full correspondence:: Prof. Carsten Watzl, Leibniz Research Centre for Working Environment and Human Factors (IfADo) at TU Dortmund, Ardeystrasse 67, 44139 Dortmund, Germany.
e-mail: watzl@ifado.de

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