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Characterization of the SARS-CoV-2 Neutralization Potential of COVID-19–Convalescent Donors

Bernd Jahrsdörfer,*† Rüdiger Groß,‡ Alina Seidel,§ Lukas Wettstein,† Carolin Ludwig,*† Tatjana Schwarz,§ Sixten Körper,*† Markus Rojewski,*† Ramin Lotfi,*† Christoph Weinstock,*† Erhard Seifried,‡ Victor Max Corman,§ Christian Drosten,§ Jan Münch,† and Hubert Schrezenmeier*†

The current SARS-CoV-2 pandemic has triggered the development of various SARS-CoV-2 neutralization tests. A wild-type virus (using African green monkey VeroE6 cells), a pseudovirus (using human Caco-2 cells), and a surrogate neutralization test platform were applied to characterize the SARS-CoV-2 neutralization potential of a cohort of 111 convalescent plasma donors over a period of seven months after diagnosis. This allowed an in-depth validation and assay performance analysis of these platforms. More importantly, we found that SARS-CoV-2 neutralization titers were stable or even increased within the observation period, which contradicts earlier studies reporting a rapid waning of Ab titers after three to four months. Moreover, we observed a positive correlation of neutralization titers with increasing age, number of symptoms reported, and the presence of the Rhesus Ag RhD. Validation of the platforms revealed that highest assay performances were obtained with the wild-type virus and the surrogate neutralization platforms. However, our data also suggested that selection of cutoff titers had a strong impact on the evaluation of neutralization potency. When taking strong neutralization potency, as demonstrated by the wild-type virus platform as the gold standard, up to 55% of plasma products had low neutralization titers. However, a significant portion of these products were overrated in their potency when using the surrogate assay with the recommended cutoff titer. In summary, our study demonstrates that SARS-CoV-2 neutralization titers are stable for at least seven months after diagnosis and offers a testing strategy for rapid selection of high-titer convalescent plasma products in a biosafety level 1 environment. The Journal of Immunology, 2021, 206: 2614–2622.

With the beginning of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic in early 2020, an unprecedented development of various molecular and serological diagnostic platforms on the one hand, as well as prophylactic and therapeutic approaches against the virus on the other hand, were initiated all over the globe. With the advent of efficient and safe vaccinations against SARS-CoV-2 (1–3) a strong need arises for the detection of SARS-CoV-2–specific Abs as well as for assays that allow quantification of virus-neutralizing potency. Respective neutralization test platforms should be characterized by rapid availability, low safety level restrictions, and a high level of assay performance and throughput. It is of paramount interest for the future development and eventual control of the pandemic to not only know how rapidly and efficiently vaccinated individuals build up but also how long they keep a sufficiently strong immunity against SARS-CoV-2. This may have implications for both the health of the vaccinated persons themselves, and also for a potential further spread or containment of the virus.

An additional area of requirement for neutralization tests are the various COVID-19–convalescent plasma donor programs, which have been launched to support the treatment of patients with a severe course of COVID-19. Although the potential benefit of convalescent plasma for the treatment of severely ill COVID-19 patients is still under debate (Ref. 4; M. J. Joyner, J. W. Senefeld, S. A. Klassen, J. R. Mills, P. W. Johnson, E. S. Theel, C. C. Wiggins, K. A. Bruno, A. M. Klompas, E. R. Lesser, et al. manuscript posted on medRxiv, DOI: 10.1101/2020.08.12.20169359; and R. Libster, G. P. Marc, D. Wappner, S. Covello, A. Bianchi, V. Braem, I. Esteban, M. T. Caballero, C. Wood, M. Berrueta, et al. manuscript posted on medRxiv, DOI: 10.1101/2020.11.20.20234013), the largest retrospective study

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B.J. and R.L. acquired and screened COVID-19–reconvalescent donors for the CAPSID trial; B.J. supervised the analytics and generated hypotheses; R.G., A.S., L.W., C.L., and T.S. performed the analytics; B.J., R.G., and C.L. conducted data analyses and prepared figures; V.M.C., S.K., M.R., R.L., C.W., E.S., C.D., J.M., and H.S. provided key research tools; S.K., E.S., and H.S. developed the CAPSID protocol; B.J. and H.S. wrote the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ACE2, angiotensin-converting enzyme 2; BSL, biosafety level; FluC, fully luciferase; NAT, nucleic acid testing; NCP, nucleocapsid protein; NT50, Ab titer required for 50% virus neutralization; PRNT, plaque reduction neutralization test; PRNT50, PRNT of 50%; PRNT90, PRNT of 90%; RBD, receptor binding domain; RhD, Rhesus Ag D; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; VSV, vesicular stomatitis virus.

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published so far in the United States, including more than 35,000 patients, demonstrated a benefit for patients receiving plasma with higher titers of SARS-CoV-2–specific Abs compared with plasma with lower titers (M. J. Joyner et al., manuscript posted on medRxiv, DOI: 10.1101/2020.08.12.20169359). In early 2020, we also initiated a national COVID-19–convalescent plasma donor program under the auspices of the German Red Cross Blood Donation Service. Within this program, we started screening patients who recovered from mild to moderate COVID-19 to evaluate their suitability as convalescent plasma donors within a prospective randomized clinical trial (CAPSID; 2020-01310-38).

Using serum samples from the growing cohort of convalescent plasma donors for this study, we validated and compared three different neutralization test platforms with distinct functionalities (Refs. 5–8). The first platform is based on direct detection of a reduction of wild-type virus–induced cell culture plaques and can currently be considered as the gold standard in this area (5, 9). The main disadvantages of this platform are the need for a biosafety level (BSL) 3 environment, because of the use of wild-type SARS-CoV-2, and a minimum time of five days to obtain results. The second platform tested is a pseudovirus neutralization test based on vesicular stomatitis virus (VSV; ΔVSV-G) pseudotyped with SARS-CoV-2 spike protein. The main advantage of this platform is that it can be performed in a BSL1 environment and delivers results after 16 hours by measuring expression of the luciferase or eGFP reporter (10). Moreover, the system can be readily adapted to evaluate neutralization efficiencies against novel SARS-CoV-2 spike variants. The third platform is a high-throughput surrogate neutralization test, which can be performed in a BSL1 environment and which delivers results within less than four hours. Its basic principle is the blocking of rHRP–labeled rSARS-CoV-2–spike receptor binding domain (RBD) molecules by neutralizing Abs, followed by a readout based on the binding of unbound HRP–labeled RBD to recombiant angiotensin-converting enzyme 2 (ACE2) protein immobilized on a microtiter plate (6). ACE2 is the main receptor for cellular uptake of SARS-CoV-2 (11).

The current study is the first, to our knowledge, characterizing the SARS-CoV-2 neutralization potential of a cohort of 111 COVID-19–convalescent plasma donors over a period of 7 mo using three different neutralization test platforms. In parallel, our study provides SARS-CoV-2 neutralization potential of a cohort of 111 COVID-19–convalescent plasma donors over a period of 7 mo using three different neutralization test platforms.

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Materials and Methods

Human subjects and serum samples

The use of blood from healthy human subjects and from COVID-19–convalescent subjects as described in the Results section was approved by the Institutional Review Board at Ulm University. For serologic and neutralization testing, 6 ml of blood from each donor was collected in serum collection tubes (VACUETTE; Greiner Bio-One, Frickenhausen, Germany) after informed consent was given. Collection tubes were centrifuged at 1500 × g and 20°C for 15 min, aliquoted into 250–1000 μl aliquots in 2-ml cryopreservation tubes (Greiner Bio-One, Frickenhausen, Germany) and cryopreserved at −20°C until further use. For long-term storage, cryopreservation tubes were transferred to −80°C after 1–5 d.

ELISA (Euroimmun)

The Euroimmun anti–SARS-CoV-2 assay is a classical ELISA for the detection of IgG in the S1 domain of the SARS-CoV-2 spike protein (S), IgA in the S1 domain of the SARS-CoV-2 spike protein, and IgG in the SARS-CoV-2 nucleocapsid protein (NCP). The assay was performed manually, according to the manufacturer’s instructions. Briefly, serum or plasma samples were diluted at 1:101 in sample buffer and pipetted onto strips of eight single wells of a 96-well microtiter plate and precoated with rSARS-CoV-2 spike or NCP. A calibrator, a positive control, and a negative control were carried out on each plate. After incubation for 60 min at 37°C, wells were washed three times, and the peroxidase-labeled anti-IgG or anti-IgA Ab solution was added, followed by a second incubation step for 30 min. After three additional washing steps, substrate solution was added, and the samples were incubated for 15–30 min in the dark. After adding the stop solution, OD values were measured on a POLARstar Omega plate reader (BMG LABTECH, Ortenberg, Germany) at 450 nm and 620 nm. Finally, OD ratios were calculated based on the sample and calibrator OD values. For all analytes, a ratio <0.8 was considered to be nonreactive or negative. An OD ratio ≥1.1 was considered to be positive for all three analytes.

Plaque reduction neutralization test for SARS-CoV-2

Plaque reduction neutralization tests (PRNT) for SARS-CoV-2 were performed as previously described for MERS-CoV (5). Briefly, VeroE6 cells (3.25 × 10⁵ cells/ml) were seeded in 24-well plates and incubated overnight. Prior to PRNT, patient sera were heat-inactivated at 56°C for 30 min. For each dilution step (duplicate), patient sera were diluted in 220 μl of Opti-MEM 1:1 with ΔVSV-G. The 440-μl serum–virus solution was gently vortexed and incubated at 37°C for 1 h. Each 24-well plate was incubated with 200 μl of serum–virus solution. After 1 h at 37°C, supernatants were discarded, and cells were supplemented with 1.2% Avicel solution in DMEM. After 3 d at 37°C, supernatants were removed, and the 24-well plates were fixed and inactivated using a 6% formaldehyde/PBS solution and stained with crystal violet, as described (9). Serum dilutions with a PRNT of 50% (PRNT50) and 90% (PRNT90) are referred to as titers. Unless stated otherwise, cutoff titers were set at 1:10.

Surrogate SARS-CoV-2 neutralization test

This blocking ELISA qualitatively detects anti–SARS-CoV-2 Abs suppressing the interaction between the RBD of the viral spike glycoprotein (S) and the ACE2 protein on the surface of cells (12, 13). Before preincubution of samples and controls, which allows Abs in the serum to bind to an HRP–conjugated RBD fragment (HRP–RBD), the mixture is added to a capture plate coated with human ACE2 protein. Any unbound HRP–RBD or HRP–RBD bound to nonneutralizing Abs is captured on the plate. Complexes of neutralizing Abs and HRP–RBD do not bind on the plate and are removed after three washing steps. Then, TMB is added as a substrate, allowing HRP to catalyze a color reaction. The color of the solution changes from colorless to yellow after addition of the stop reagent, which can be read by a microtiter plate reader at an OD of 450 nm (OD450). The absorbance of the sample is inversely correlated with the amount of SARS-CoV-2 neutralizing Abs. Positive and negative controls serve as internal assay quality controls; the test is considered valid only if the OD450 for each control falls within the respective range (OD450negative control > 1.0, OD450positive control < 0.3). For final interpretation, the inhibition rates were determined using the following formula: Inhibition rate (%) = (OD valuecontrol − OD valueneutralizing control) × 100%). Unless stated otherwise, scores <20% were considered negative; scores ≥20% were considered positive.

Pseudovirus neutralization test

This neutralization test can be performed under BSL1 conditions using firefly luciferase (Fluc)-CoV-2–spike pseudoviruses based on a VSV strain encoding eGFP and Fluc in place of its native glycoprotein, designated VSVΔG(eGFP,Fluc) (ΔVSV-G). The VSV pseudovirus constructs are kindly provided by G. Zimmer, Institute of Virology and Immunology, Mittelhauzen, Switzerland. Readout can be performed by flow cytometry or luminescence measurement as described (10). Briefly, SARS-CoV-2 spike pseudoviruses are produced on HEK293 T cells transfected on day 1 with a plasmid encoding SARS-CoV-2 spike protein. On the next day, the cells are transfected with VSV-G pseudovirus to deliver the viral reporter genome. During the second day, the particles are harvested and concentrated via ultrafiltration. To test the neutralizing potential of serum from SARS-CoV-2–convalescent individuals, the serum is serially diluted (up to a concentration of 10%), then incubated with SARS-CoV-2 spike pseudovirus and finally added to Caco-2 cells at a 1:10 dilution and cultivated for 16 h. For readout, Fluc activity in Caco-2 cells is measured using the Promega Luciferase Assay System (catalog no. E1501) and normalized using cells transduced with spike protein–expressing pseudoviruses incubated in PBS only. Results are given as the Ab titer required for 50% virus neutralization (NT50), representing the dilution of serum reducing the transduction of Caco-2 cells by 50%. Unless stated otherwise, the cutoff value of this assay was set at NT50 < 10.

Statistical analysis

Summarized data are generally expressed as means ± SEM. Statistical differences between the means of two data sets were assessed using the unpaired, two-sided Student t test. Correlations between two data sets were calculated using Pearson correlation coefficient and the r distribution with n − 2 degrees of freedom
freedom. The $p$ values $<0.05$ were considered to indicate statistical significance; $p$ values $<0.005$ were considered to indicate high statistical significance.

Results

Characteristics of test cohorts

Serum samples were collected from up to 111 individuals (a complete data set was available for 96 individuals) who presented to our institute for assessment as potential convalescent plasma donors for a randomized prospective trial of convalescent plasma for the treatment of patients with severe COVID-19 (CAPSID; EudraCT 2020-001310-38). All donors had a history of SARS-CoV-2 infection (COVID-19+) confirmed by a positive nucleic acid testing (NAT) test result from a nasopharyngeal swab. COVID-19 courses of these donors were characterized by the presence of rather mild to moderate symptoms, including loss of taste and/or olfaction, limb pain and headache, fever up to 40°C, dry cough, fatigue, congested nose, sore throat, chills, loss of appetite, diarrhea, and shortness of breath. A median of three different symptoms was reported; three individuals reported no symptoms at all, and one individual reported more severe symptoms, which required oxygen support and hospitalization. Gender distribution and frequency of symptoms for the convalescent plasma donor cohort are summarized in Table I. The average age was 40 y (20–61 y), 51.0% were males, 49.0% were females. Median duration between symptom onset and serology was 50 d (18–170 d), median duration of the symptomatic period was 12 d (0–154 d), median duration from documented positive pharyngeal swab SARS-CoV-2 NAT to serology was 49 d (10–165 d), and median duration from symptom convalescence to serology was 36 d (5–143 d).

Serum samples for the negative control cohort (COVID-19-) were collected from up to 102 healthy individuals with no reported COVID-19-suspicious symptoms since January 2020, no contact to COVID-19 patients, or a negative pharyngeal swab SARS-CoV-2 NAT result in the suspected presence of either risk contacts or COVID-19 patients, or a negative pharyngeal swab SARS-CoV-2 NAT result in the suspected presence of either risk contacts or symptoms. In addition, all subjects of the negative control cohort were tested negative for anti–nucleocapsid–IgG as well as for anti–SARS-CoV-2 spike–IgG and IgA (Fig. 1A). Median age in the control cohort was 49 y (20 to 63 y), 27.1% were males, 72.9% were females.

Assay performance indicators of neutralization test platforms

A total of 192 serum samples (96 COVID-19+ and 96 COVID-19- samples) were tested on three different neutralization test platforms, including the following: 1) a PRNT based on wild-type SARS-CoV-2 as the gold standard (PRNT50 and PRNT90), 2) an Ab titer required for 50% virus neutralization (NT50) based on a VSV-based pseudovirus, and 3) a surrogate neutralization test based on the inhibition of SARS-CoV-2 RBD interaction with the ACE2 protein. Semiquantitative analysis confirmed that the results from all platforms evaluated (PRNT50 and PRNT90, pseudovirus NT50, and ACE2 ELISA inhibition) were significantly higher in the COVID-19+ compared with the COVID-19- cohort (Fig. 1A–C). Moreover, our data suggest that older individuals (Fig. 2) and individuals with a higher number ($n > 3$) of symptoms developed higher neutralization titers against wild-type SARS-CoV-2 than younger individuals and individuals with a lower number ($n \leq 3$) of symptoms (Supplemental Fig. 1A–D). No difference in neutralization responses were found between male and female donors (Suppl. Fig. 1E–H).

Qualitative analysis of neutralization test results (positive or negative, based on the recommended cutoff values) allowed us to calculate basic test parameters, including sensitivity, specificity, and positive and negative predictive values (Supplemental Table 1). The results of this analysis, however, must be interpreted with caution because they are based on the idealized assumption that all COVID-19–positive individuals develop neutralizing anti–SARS-CoV-2 Abs, whereas all COVID-19–negative individuals do not. Because, presumably, this does not accurately reflect the real situation, we performed an additional subgroup analysis after splitting the COVID-19–positive cohort into one subcohort (PRNT90+) with strong neutralizing capacity (90% inhibition plaque reduction test $\geq 1:20$) and one subcohort (PRNT90-) without strong neutralizing capacity (90% inhibition plaque reduction test $<1:20$) (Table II). The results of this subgroup analysis demonstrated that highest sensitivities were obtained with the wild-type virus PRNT50 using cutoff titers between 1:20 and 1:80, the ACE2 ELISA from GenScript using the recommended cutoff value of 20%, and the pseudovirus neutralization testing using the recommended cutoff value of 10 (Table II). Specificity, in contrast, was very low for all platforms when the recommended cutoff values were used. When cutoff values were gradually increased, specificity was highest with the ACE2 ELISA from GenScript (100%) at a cutoff value of 80%, followed by a specificity of 97.9% both with the wild-type virus PRNT50 and the pseudovirus neutralization test at cutoff titers of 1:160 and 25, respectively (Table II). For comparison, we included a similar analysis for the anti–SARS-CoV-2 spike–IgG ELISA platform. Although its sensitivity rapidly decreased by increasing the cutoff values, the use of a cutoff OD ratio of 5.0 was almost equivalent for identifying sera with high neutralization titers as compared with the ACE2 ELISA (Table II, bottom row).

Correlations and concordances between neutralization testing platforms

When correlating the results from the wild-type virus PRNT50 with the pseudovirus neutralization test platform (Fig. 3A), the surrogate neutralization test platform (Fig. 3B), and the serologic results from the anti–SARS-CoV-2 spike–IgG ELISA platform from Euroimmun (Fig. 3C), highly significant correlations were found for all three analytes. Of note, all three surrogate analytes delivered false positive results, particularly the ACE2 ELISA from GenScript (Fig. 3A–C, first columns on the left

Table I. Symptoms of convalescent plasma donors

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Loss of taste / olfaction</th>
<th>Limb pain / headache</th>
<th>Fever</th>
<th>Dry cough</th>
<th>Fatigue</th>
<th>Congested nose</th>
<th>Sore throat</th>
<th>Chills</th>
<th>Loss of appetite</th>
<th>Diarrhea</th>
<th>Shortness of breath</th>
<th>No symptoms</th>
<th>Hospitalization / Oxygen support</th>
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<tr>
<td>Absolute number ($n=96$)</td>
<td>69</td>
<td>58</td>
<td>49</td>
<td>47</td>
<td>36</td>
<td>25</td>
<td>20</td>
<td>13</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Frequency (%)</td>
<td>71.9%</td>
<td>60.4%</td>
<td>51.0%</td>
<td>49.0%</td>
<td>37.5%</td>
<td>26.0%</td>
<td>20.8%</td>
<td>13.5%</td>
<td>11.5%</td>
<td>11.5%</td>
<td>11.5%</td>
<td>11.5%</td>
<td>11.5%</td>
</tr>
<tr>
<td>Male ($n=49$)</td>
<td>35</td>
<td>35</td>
<td>24</td>
<td>28</td>
<td>21</td>
<td>13</td>
<td>10</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>4</td>
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<td>0</td>
</tr>
<tr>
<td>Frequency (51.0%)</td>
<td>71.4%</td>
<td>71.4%</td>
<td>49.0%</td>
<td>57.1%</td>
<td>42.9%</td>
<td>26.5%</td>
<td>20.4%</td>
<td>12.2%</td>
<td>14.3%</td>
<td>12.2%</td>
<td>8.2%</td>
<td>2.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Female ($n=47$)</td>
<td>34</td>
<td>23</td>
<td>15</td>
<td>15</td>
<td>12</td>
<td>10</td>
<td>7</td>
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<td>5</td>
<td>7</td>
<td>1</td>
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<td>1</td>
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<tr>
<td>Frequency (49.0%)</td>
<td>72.3%</td>
<td>48.9%</td>
<td>53.2%</td>
<td>40.4%</td>
<td>31.9%</td>
<td>25.5%</td>
<td>21.3%</td>
<td>14.9%</td>
<td>8.5%</td>
<td>10.6%</td>
<td>14.9%</td>
<td>4.3%</td>
<td>2.1%</td>
</tr>
</tbody>
</table>

Information on symptoms was available from 96 plasma donors after convalescence from mild to moderate COVID-19 disease as documented by both positive pharyngeal swab NAT and positive anti–SARS-CoV-2-spike–IgG test results.
The correlations between the PRNT50 results and anti-SARS-CoV-2 spike–IgG and anti-SARS-CoV-2 nucleoprotein–IgG, respectively, were also strong but at a lower level of significance (data not shown). This is in line with our recent side-by-side validation of the abovementioned serological analyses, which also demonstrated the most significant correlation between the anti–spike–IgG and the PRNT50 results (7). We therefore included correlation analysis between the anti–spike–IgG ELISA and the pseudovirus as well as the surrogate neutralization test platforms, demonstrating high correlations between the results from both platforms and anti–spike–IgG titers (Fig. 3D, 3E). Matching these results, the overall concordance rates between the wild-type neutralization test platform and the alternative platforms were also high, reaching >96% for the surrogate neutralization test from GenScript and >86% for the pseudovirus neutralization test (Supplemental Table II).

**FIGURE 1.** Anti-SARS-CoV-2 spike–IgG and neutralization titers in COVID-19 convalescent patients, versus healthy control subjects. Serum samples from 96 plasma donors after convalescence from mild to moderate COVID-19 as documented by positive pharyngeal swab NAT and positive anti-SARS-CoV-2 spike–IgG test results (COVID-19–positive) as well as from 96 anti-SARS-CoV-2 spike–IgG–negative healthy subjects (COVID-19–negative) with either no COVID-19 symptoms and no risk contacts or with a negative pharyngeal swab NAT testing, were collected. Subsequently samples were tested using (A) an anti-SARS-CoV-2 spike–IgG ELISA, (B) an anti-SARS-CoV-2 neutralization test based on a VSV-based pseudovirus, and (C) a surrogate neutralization test based on the inhibition of SARS-CoV-2 RBD interaction with the ACE2 protein. Error bars indicate SEM. The \( p \) values <0.001 indicate highly significant differences.

**FIGURE 2.** Correlation between wild-type virus SARS-CoV-2 neutralization titers and donor age. Serum samples from 96 convalescent plasma donors after mild to moderate COVID-19 disease, as documented by both positive pharyngeal swab NAT and positive anti-SARS-CoV-2 spike–IgG test results that were collected. Subsequently, samples were tested using (A) the wild-type virus PRNT50, (B) the wild-type virus PRNT90, (C) a neutralization test based on a VSV-based pseudovirus, and (D) a surrogate neutralization test based on the inhibition of SARS-CoV-2 RBD interaction with the ACE2 protein. Results were correlated with donor age. Error bars indicate SEM. The \( p \) values <0.05 indicate significant; \( p \) values <0.005 indicate highly significant Pearson correlations.
Serum samples from 48 anti–SARS-CoV-2 spike–IgG–positive COVID-19–convalescent plasma donors with PRNT90 neutralization titers >1:20 (PRNT90+) and from 48 anti–SARS-CoV-2 spike–IgG–positive COVID-19–convalescent plasma donors with PRNT90 neutralization titers <1:20 (PRNT90−) were collected. Subsequently, samples were tested using the following: 1) the wild-type virus PRNT50, 2) a neutralization test based on a VSV-based pseudovirus, (3), a surrogate neutralization test based on the inhibition of SARS-CoV-2 RBD interaction with the ACE2 protein, and 4) an ELISA for anti–SARS-CoV-2 spike–IgG. Analytic results were calculated based on increasing cutoff values as indicated.

### Analysis of serological and neutralization responses over time

For the establishment of a COVID-19–convalescent plasma donor program, it is of particular interest to know the time courses of serological anti–SARS-CoV-2 Ab responses in general and neutralization responses in particular. Up to the date of submission, we were able to analyze a total of 269 serum samples from 111 individual convalescent plasma donors over a period of 7 mo after positive pharyngeal swab NAT. During this time, 54 donors presented for one and 57 donors for two or more appointments. Overall, the correlations between the time from diagnosis to analysis of the different analytes were rather weak but nevertheless reached statistical significance in some cases. Although the average anti–SARS-CoV-2 nucleocapsid–IgG response (Supplemental Fig. 2A) decreased from an OD ratio of 3.4 to 1.4 and the anti–SARS-CoV-2 spike–IgA response decreased (Supplemental Fig. 2B) from 3.1 to 1.7 between days 10 and 215 after symptom onset, the average anti–SARS-CoV-2 spike–IgG response rather increased from an OD ratio of 3.4 to 5.8 during the same time period (Supplemental Fig. 2C). These increasing anti–spike–IgG titers were paralleled by the neutralization titers obtained with the surrogate ACE2 ELISA from GenScript (Fig. 4A, 4B). Importantly, also the PRNT confirmed that the wild-type neutralization capacity of the tested sera remained stable within the observation time of 7 mo (Fig. 5C, 5D).

<table>
<thead>
<tr>
<th>Platforms</th>
<th>Validation groups</th>
<th>Wildtype virus plaque reduction neutralisation test (PRNT50)</th>
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<tr>
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<td>Cutoff values</td>
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<td></td>
<td>Positive results (&quot;true&quot;</td>
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### Table II. Subgroup analysis and impact of cutoff values on assay performance indicators of different SARS-CoV-2 Ab and neutralization test platforms
![FIGURE 3. Correlations between serologic anti–SARS-CoV-2 spike–IgG as well as pseudovirus, surrogate and wild-type virus SARS-CoV-2 neutralization titers. A total of 269 serum samples from 111 individual convalescent plasma donors after mild to moderate COVID-19 disease as documented by a positive pharyngeal swab NAT test result (red circles) that were collected. Subsequently samples were tested using the wild-type virus PRNT50, a surrogate neutralization test based on the inhibition of SARS-CoV-2 RBD interaction with the ACE2 protein, and a neutralization test based on a VSV-based pseudovirus. The control group (gray circles) consisted of serum samples from 102 healthy subjects with either no COVID-19 symptoms and no risk contacts or with a negative pharyngeal swab NAT test result. (A–C) The wild-type virus PRNT50 titers from 111 individual convalescent plasma donors were correlated with (A) the results from the pseudoparticle neutralization test, (B) the results from the surrogate ACE2 ELISA from GenScript, and (C) the serologic anti–SARS-CoV-2 spike–IgG ELISA results. Cutoff values are indicated by dotted lines. (D and E) The results from the anti–SARS-CoV-2-spine-IgG ELISA were correlated with (D) the results from the pseudovirus neutralization test and (E) the results from the surrogate neutralization test from GenScript. The p values <0.0001 indicate highly significant Pearson correlations.

**Association between blood group Ags and neutralization responses**

Finally, we wished to evaluate potential associations between various blood group Ags and the development of SARS-CoV-2 neutralization responses in COVID-19–convalescent individuals. Inspired by the observation that naturally occurring isoagglutinins may influence the anti-glycan immune response against enveloped viruses, such as SARS-CoV-2 (14), we first compared the SARS-CoV-2 neutralization responses in donors with different ABO blood groups. Although we found consistent trends for stronger SARS-CoV-2 neutralization titers in subjects not carrying the isoagglutinin A (blood groups A and AB) as compared with subjects carrying anti-A (blood groups O and B), this trend did not reach statistical significance (Fig. 5A). In contrast, we detected a significant and so far, to our knowledge, undescribed trend to higher SARS-CoV-2 neutralization responses in Rhesus Ag D (RhD)–positive as compared with RhD-negative donors (Fig. 5B). No differences were found for Kell+ versus Kell individuals (Fig. 5C).

**Discussion**

Currently there is a great heterogeneity worldwide with regard to test platforms used for the identification of suitable plasma products for COVID-19–convalescent plasma donor programs. In most studies, surrogate serological test systems are used instead of neutralization test platforms. For example, a large open-label trial for the treatment of COVID-19 patients with human convalescent plasma involving over 35,000 patients in the United States used the serological chemiluminescent immunoassay platform VITROS from Ortho Clinical Diagnostics, which allows the detection of anti–SARS-CoV-2 spike–IgG (M. J. Joyner et al. manuscript posted on medRxiv, DOI: 10.1101/2020.08.12.20169359). Similarly, in two prospective, randomized trials of convalescent plasma in patients with severe COVID-19 in Argentina, the potency of convalescent plasma products was determined with an alternative serological anti–SARS-CoV-2 spike–IgG test system but not with a platform measuring the actual virus neutralization potential (Ref 4 and R. Libster et al., manuscript posted on medRxiv, DOI: 10.1101/2020.11.20.20234013). Of note, the serological platforms used in the Argentinian trials were initially correlated with the same surrogate neutralization test platform from GenScript (ACE2 ELISA) that we evaluated in our present study. As described above, we found that this assay provides an excellent sensitivity that is comparable with the wild-type virus platform. Its specificity, however, turned out to be extremely low when the recommended cutoff value was used, resulting in a high number of false positive results, which means an overestimation of the neutralization potency of potential convalescent plasma products. In line with data from other groups (15), our national convalescent plasma program experience suggests that up to 55.4% of all donated convalescent plasma products contain wild-type virus PRNT50 titers <1:80 and therefore have to be considered as low-titer products. The fact that the Argentinian studies failed to find a significant benefit in the convalescent plasma-treated as compared with the placebo-treated group of patients may therefore, at least in part, be due to the use of a high number of insufficiently neutralizing convalescent plasma products (Ref 4, and R. Libster et al., manuscript posted on medRxiv, DOI: 10.1101/2020.11.20.20234013).

Our validation revealed that a gradual increase of the recommended cutoff values resulted in an improvement of the assay performance parameters for some neutralization platforms. For example, for the wild-type neutralization test platform, a specificity of 75%...
could be reached without loss of the maximal sensitivity when the cutoff titer was set at 1:80. For the surrogate neutralization test from GenScript, specificity could be increased to 100% when a cutoff value of 80% was used. This, however, resulted in a reduction of the sensitivity to 70.8%. Although this sensitivity is not optimal, it may be outweighed by the ease and rapidity of the assay and may therefore be justifiable when the main purpose of testing is to identify highly potent convalescent plasma products, even if that approach means that a certain percentage of potentially effective products are sorted out. Almost equivalent for identifying sera with high neutralization responses in the investigated donor cohort. Initially, we evaluated the general Ab responses against SARS-CoV-2 spike and NCP. We found that the IgG response against SARS-CoV-2 spike steadily increased over the observation period of seven months after diagnosis. This appears to be in contrast with a recent report describing a waning of anti-SARS-CoV-2 RBD Ab responses in convalescent plasma donors within four months after symptom onset (16). Nevertheless, the data presented in the study by Perreault and colleagues (16) are in line with our finding that IgA titers against SARS-CoV-2 spike as well as IgG titers against SARS-CoV-2 nucleocapsid also decreased over time, with the strongest loss of titers between the first and the third month after diagnosis. Support for our findings also comes from three additional independent studies that not only observed the development of a stable humoral immune response against the SARS-CoV-2 spike protein between three and five months after SARS-CoV-2 infection (17, 18), but also observed the occurrence

FIGURE 4. Time course of SARS-CoV-2 neutralization titers. Serum samples from 111 individual convalescent plasma donors after mild to moderate COVID-19 disease and from 269 appointments altogether were collected at different time points after positive pharyngeal swab NAT testing. Subsequently samples were analyzed using the surrogate neutralization test from GenScript (A and B) and the wild-type virus PRNT (C and D). A total of 54 donors presented for one appointment; 57 donors presented for two or more appointments. Line graphs show time courses based on the time period from positive pharyngeal swab testing to analysis or from the end of symptoms to analysis. Trend lines indicate average time courses of the titers. The p values <0.05 indicate significance; p values <0.005 indicate highly significant Pearson correlations.

PRNT50 at a cutoff titer of 1:80 and with the ACE2 ELISA from GenScript at a cutoff value of 80%.

The present study put a special focus on the temporal evaluation of anti–SARS-CoV-2 Ab and neutralization responses in the investigated donor cohort. Initially, we evaluated the general Ab responses against SARS-CoV-2 spike and NCP. We found that the IgG response against SARS-CoV-2 spike steadily increased over the observation period of seven months after diagnosis. This appears to be in contrast with a recent report describing a waning of anti–SARS-CoV-2 RBD Ab responses in convalescent plasma donors within four months after symptom onset (16). Nevertheless, the data presented in the study by Perreault and colleagues (16) are in line with our finding that IgA titers against SARS-CoV-2 spike as well as IgG titers against SARS-CoV-2 nucleocapsid also decreased over time, with the strongest loss of titers between the first and the third month after diagnosis. Support for our findings also comes from three additional independent studies that not only observed the development of a stable humoral immune response against the SARS-CoV-2 spike protein between three and five months after SARS-CoV-2 infection (17, 18), but also observed the occurrence
of corresponding memory B cells reaching a peak at six months postinfection (19). Interestingly, the results from the surrogate neutralization test from GenScript paralleled those of the anti-SARS-CoV-2 spike IgG titers, whereas the results from the wild-type virus platform suggested a relatively stable neutralization capacity that appeared to be independent from the course of the serological results. All these seemingly contradictory results may be a hint for the existence of additional routes for the virus to infect cells apart from the well-described pathway that involves interaction between the SARS-CoV-2 RBD and ACE2 (11). As an example in this context, a very recent study suggested the existence of coreceptors, such as neuropilin-1 (BDCA4), which may facilitate SARS-CoV-2 cell entry and infectivity (20). It is known that most Abs produced against SARS-CoV-2 are not binding to the RBD of the spike protein (21). It may well be possible, therefore, that the neutralization capacity of patient serum and convalescent plasma involves additional Ab specificities that remain undetected using the currently available commercial kits.

An interesting side finding of the current study was that COVID-19 convalescent donors carrying the RhD mounted significantly stronger SARS-CoV-2 neutralization responses than RhD-negative donors. This finding matches observations from a recent publication demonstrating that subjects with anti-A in their serum are significantly underrepresented among COVID-19 patients compared with subjects without anti-A (23). Moreover, the observation that interaction between the spike protein of the closely related SARS-CoV and its main entry protein ACE2 is blocked by human natural anti-A Abs (24) suggests that the natural anti-glycan immune response represented by human isoagglutinins may limit the transmission of enveloped viruses such as SARS-CoV-2 (14).

One parameter often discussed in connection with COVID-19 susceptibility is patient age. Although the course of COVID-19 is generally more severe with increasing age (25, 26), it remains unclear, so far, whether susceptibility or severity of COVID-19 depends on various neutralizing Ab responses. However, if neutralizing Abs are to play a major role for susceptibility or severity, a more pronounced and efficient humoral immune response upon initial contact to SARS-CoV-2 would be expected in younger compared with older patients.
individuals. Unlike what is expected, however, our data suggested the opposite because we found a moderate but highly significant positive correlation between age and neutralizing Ab titers. Therefore, this study and also further studies with larger cohorts, which optimal-ly will also include an evaluation of the cellular anti--SARS-CoV-2 immune response (27), are necessary to shed more light into this par-ticular question.

In summary, the current study characterizes the SARS-CoV-2 neutralization potential and its temporal course in up to 111 conva-lescent plasma donors. Using three functionally independent neutral-ization test platforms, we found that neutralization titers appeared to be stable or even increase over a time period of seven months after diagnosis. We also found that neutralization titers were stronger with increasing donor age, with a higher number of symptoms and in the presence of the RhD, although these associations were not strong enough to justify their use for the selection of convalescent plasma donors. More importantly, our study provides an in-depth validation of the three neutralization test platforms used, which in-cluded a wild-type virus-based, a pseudovirus-based, and a surrogate neutralization test platform based on the interaction of the SARS-CoV-2 RBD with ACE2. Our validation revealed that, depending on the selection of cutoff values or titers, acceptable assay performance parameters can be obtained with all test systems. However, when taking into consideration the strong neutralization potency as demonstrated by the wild-type virus platform (PRNT90) as the gold standard, the highest combined assay performances were obtained with the wild-type virus PRNT50 at a cutoff titer of 1:80 and with the surrogate neutralization platform at a cutoff value of 80%. The data presented in this study may be used to implement a robust test-in-g strategy for rapid selection of high-titer convalescent plasma products and possibly for the screening of individuals vaccinated against SARS-CoV-2 in a BSL1 environment.

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Disclosures

V.M.C. is named together with Euroimmun on a patent application filed re-cently regarding the diagnosis of SARS-CoV-2 by IgA testing. The other au-thors have no financial conflicts of interest.

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