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MAJOR ARTICLE

Anti-Severe Acute Respiratory Syndrome Coronavirus 2 Hyperimmune Immunoglobulin Demonstrates Potent Neutralization and Antibody-Dependent Cellular Cytotoxicity and Phagocytosis Through N and S Proteins

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Background. Although coronavirus disease 2019 (COVID-19) vaccinations have provided a significant reduction in infections, effective COVID-19 treatments remain an urgent need.

Methods. Functional characterization of anti-severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) hyperimmune immunoglobulin (hIG) from human convalescent plasma was performed by different virus neutralization methodologies (plaque reduction, virus-induced cytotoxicity, median tissue culture infectious dose [$TCID_{50}$] reduction, and immunofluorimetry) at different laboratories using geographically different SARS-CoV-2 isolates (USA [1], Italy [1], and Spain [2]; 2 containing the D614G mutation). Neutralization capacity against the original Wuhan SARS-CoV-2 strain and variants (D614G mutant, B.1.1.7, P.1, and B.1.351) was evaluated using a pseudovirus expressing the corresponding spike (S) protein. Antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) was also evaluated.

Results. All SARS-CoV-2 isolates were potently neutralized by hIG as shown by all 4 methodologies. Wild-type SARS-CoV-2 and variants were effectively neutralized using the pseudovirus. The hIG (IgG type) induced ADCC and ADCP against SARS-CoV-2 N and S proteins but not E protein. Very low concentrations (25–100 µg IgG/mL) were required. A potent effect was triggered by antibodies in hIG solutions against the SARS-CoV-2 S and N proteins.

Conclusions. Beyond neutralization, IgG Fc-dependent pathways may play a role in combatting SARS-CoV-2 infections using COVID-19 hIG. This could be especially relevant for the treatment of more neutralization-resistant SARS-CoV-2 variants.

Keywords. ADCC-ADCP; hyperimmune immunoglobulin; SARS-CoV-2; variants; viral neutralization.

Currently, there is no effective standardized treatment for coronavirus disease 2019 (COVID-19), although multiple therapeutic options are available [1]. Among the available therapeutic strategies, passive immunization using COVID-19 convalescent plasma (CCP), monoclonal antibodies (mAbs), or hyperimmune immunoglobulin ([hIG] immunoglobulin G [IgG] enriched with anti-severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2] antibodies) is of particular relevance [2]. As the SARS-CoV-2 pandemic spreads and vaccination progresses, commercial IgG products derived from healthy plasma donors become gradually enriched in anti-SARS-CoV-2 antibodies [3]. To date, the antibody levels in the general population are still low [3]; therefore, the plasma collected and the products produced cannot yet be considered hyperimmune.

Anti-SARS-CoV-2 hIG is typically prepared from pools of 100–1000 liters from CCP donors. The hIG products have a high titer of neutralizing antibodies against SARS-CoV-2 in a standardized and concentrated product [4]. This represents an advantage over treatment with CCP. Moreover, in contrast to mAb, hIG are polyclonal antibodies that recognize different epitopes of the virus. Key targets of anti-SARS-CoV-2 antibodies include the following: the S protein [5], responsible for viral entry through recognition of the primary host cellular receptor angiotensin-converting enzyme 2 (ACE-2); and the N protein [6], which makes up the helical nucleocapsid. The E protein, a small polypeptide, and the M protein, embedded in the envelope [7], have been less studied as potential immune targets.

More importantly, IgG possess other antiviral properties, beyond neutralization, that have been described for

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CCP. These include antigen-dependent Fc functions, eg, antibody-dependent cellular phagocytosis (ADCP) [8], antibody-dependent cellular cytotoxicity (ADCC) [9], and complement-mediated cytotoxicity [10]. These well described effector functions of antibodies (mediated by the interaction of immunoglobulin Fc with cellular Fc receptors) may add to neutralizing activity and may enable nonneutralizing antibodies or antibodies with poor-neutralizing capacity to block or clear infection. The hIG efficacy is being tested in ongoing randomized clinical trials in inpatients (intravenous administration) [11] and outpatients (subcutaneous administration) [12].

Apart from their SARS-CoV-2-neutralizing capacity, no further antiviral capacity of hIG has been experimentally demonstrated. In this study, we report an extensive functional characterization of a well characterized hIG product [4]. We performed neutralization assays on several virus isolates and on a pseudovirus expressing the most relevant S variants to date, and, for the first time, we evaluated the capacity of hIG to trigger antigen-dependent IgG Fc functions.

MATERIAL AND METHODS

Study Design

The anti-SARS-CoV-2 hIG 10% (Grifols, Barcelona, Spain) prepared from CCP [4] was functionally characterized in vitro. Neutralization of 4 geographically diverse isolates of SARS-CoV-2 were assessed by 4 different methodologies (plaque reduction, protection from virus-induced cytotoxicity, median tissue culture infectious dose [$TCID_{50}$] reduction, and immunofluorimetry-based methodology) at 4 different laboratories. The capacity of the hIG to neutralize SARS-CoV-2 variants was also evaluated using a pseudovirus test platform expressing the S proteins of the relevant variants. Finally, the capacity of hIG to induce ADCC and ADCP on the same samples and the viral protein responsible of eliciting these responses were evaluated. Positive (CCP) and negative single-donation plasmas were used for comparison.

Cell Lines and Culture

At the National Institutes of Health (NIH), Vero cells were acquired from the American Type Culture Collection ([ATCC] no. CCL-81; Manassas, VA). At CReSA-IRTA, Vero cells were obtained from the ATCC (ATCC CRL-1586). At CNB-CSIC, Vero cell lines were kindly provided by Dr. E. Snjider (University of Leiden Medical Center, Leiden, Netherlands). At Texcell, Vero cells provided by Pasteur Institut were used. At IrsiCaixa, HEK293T cells overexpressing wild-type (WT) human ACE-2 (Integral Molecular, Philadelphia, PA) were used for pseudovirus neutralization assays. Culture conditions for these cell lines are detailed in the Supplemental Information.

Severe Acute Respiratory Syndrome Coronavirus 2 Strains

Stock viruses were prepared by collecting the supernatant from Vero cells, as previously described [13]. At the NIH, SARS-CoV-2 (GenBank accession no. MT020880) was provided by the US Centers for Disease Control and Prevention ([CDC] Washington isolate; CDC, Atlanta, GA), isolated from the first US COVID-19 patient [14]. At IRTA-CReSA, SARS-CoV-2 was isolated from nasopharyngeal swab from an 89-year-old male patient from Badalona (Spain) in March 2020 (accession no. EPI ISL 418268 at GISAID repository [http://gisaid.org]) with the Spike mutations D614G, NSP12, and P323L. At CNB-CSIC, SARS-CoV-2MAD6 was isolated from nasopharyngeal swab from a 69-year-old male patient from Hospital "12 de Octubre" in Madrid (Spain). Full-length virus genome was identical to SARS-CoV-2 reference sequence (Wuhan-Hu-1 isolate; GenBank accession no. MN908947), except for the presence of a silent mutation C3037>T, and 2 mutations leading to amino acid changes: C14408>T (in nsp12) and A23403>G (D614G in S protein). At Texcell, 2019-nCoV strain 2019-nCoV/Italy-INMI1 (https://www.ncbi.nlm.nih.gov/nuccore/MT066156) isolated from the first case of COVID-19 in Italy was used [15].

Convalescent Plasmas

The SARS-CoV-2 antibody-positive plasmas were collected by plasmapheresis from CCP donors (single donation) at Grifols US plasma collection centers (Biomat USA, Inc., Interstate Blood Bank Inc., Talecris Plasma Resources, Inc.). The CCP was collected during the first half of 2020 from donors with different degrees of COVID-19 severity (mild to hospitalized).

The COVID-19-specific antibody levels in the CCP were classified as high (positive at ≥1/10 000 dilution), medium (positive at 1/1000), and low (positive at 1/100) as determined by anti-SARS-CoV-2 S enzyme-linked immunosorbent assay (ELISA) methods: human anti-SARS-CoV-2 virus spike 1 (S1) IgG ELISA Kit (Alpha Diagnostic International, Inc.), against S1 subunit spike protein; EI-2606-9601-G, Anti-SARS-CoV-2 IgG ELISA Kit (Euroimmun AG, Luebeck, Germany), against structural protein (S1 domain); DEIASL019, SARS-CoV-2 IgG ELISA Kit (Creative Diagnostics), against virus lysate.

The SARS-CoV-2 antibody-negative plasma (prepandemic collection during 2019) was used as a negative control. The CCP was used to compare responses between positive CCP samples of different positivity grades (low, medium, and high) with negative plasma controls and hIG.

Severe Acute Respiratory Syndrome Coronavirus 2 Neutralization Experiments

A previously described cell-based immunofluorescence assay (CBIFA) was used at the NIH. The details of this assay are included in the Supplemental Information. Data are reported based on a 4-parameter regression curve (using a constrained

fit) as a 50% neutralization titer (half-maximal inhibitory dilution [ID_{50}]) [4, 16].

A cytopathic-cytotoxicity luminometry assay (CCLA) was used at IRTA-CReSA. The ID_{50} values were determined from the fitted neutralization curves as the plasma dilutions that produced 50% neutralization. A summary of the assay is included in the Supplemental Information, and details of the technique are available elsewhere [17, 18].

Plaque-forming unit (PFU)-based neutralization assay was used at CNB-CSIC. Details of the assay are included as Supplemental Information. The neutralization potency of the hIG product (ID_{50} value) was expressed as plaque reduction neutralization test ($PRNT_{50}$) value, calculated as the $-\log_{10}$ of the reciprocal of the highest hIG dilution to reduce the number of plaques by 50% compared with the number of plaques without intravenous immunoglobulin (IVIG) [18].

The $TCID_{50}$-based microneutralization assay was used at Texcell. A brief summary of the assay is included as Supplemental Information. The viral titer is expressed in "dose infecting 50% of tissue cultures per mL" with a confidence interval of 95%. For neutralization plate, the ID_{50} value was expressed as the neutralization titer 50 (NT_{50}) value, calculated as the antibody titer neutralizing sample according to the Spearman-Kärber formula. The NT_{50} corresponds to the dilution of sample that prevents the cells from cytopathic effect ([CPE] no lysis) in 50% of the replicates. The criteria for the validation of the run were as follows: back titration of the virus in the $TCID_{50}$ criteria; integrity of the uninfected cells (medium control only); and absence of cell layer or presence of CPE in infected wells (virus control only).

Generation of Spike Expression Plasmids

The SARS-CoV-2.SctΔ19 Wuhan, B.1.1.7, P.1, and B.1.351 variants were generated (GeneArt) from the full protein sequence of the respective spike sequences, with a deletion of the last 19 amino acids in C-terminal [19]. Sequences were human-codon optimized and inserted into pcDNA3.1(+). The G614 spike mutant was generated by site-directed mutagenesis as previously described [20]. A summary of the methodology is included in the Supplemental Information.

Pseudovirus Generation and Neutralization Assay

In the experiments performed at the IrsiCaixa AIDS Research Institute, human immunodeficiency virus (HIV) reporter pseudovirus expressing SARS-CoV-2 S protein and Luciferase were generated using a plasmid coding for a nonreplicative HIV reporter pNL4-3.Luc.R-.E- obtained from the NIH AIDS Reagent Program [21] and the spike expression plasmids (as described above and in the Supplemental Information). The methodology is summarized in the Supplemental Information. The neutralization assay has been previously validated in a large subset of samples [22]. Neutralization assays were performed in duplicate as previously described [22]. The neutralization assay is briefly described in the Supplemental Information. The values were normalized, and the ID_{50} (the reciprocal dilution inhibiting 50% of the infection) was calculated by plotting and fitting the log of plasma dilution versus response to a 4-parameters equation in Prism 8.4.3 (GraphPad Software).

Antibody-Dependent Cellular Cytotoxicity-Antibody-Dependent Cellular Phagocytosis Induction Experiments

The ADCC- and ADCP-specific mechanisms were assayed using bioluminescent reporter assays for quantifying ADCC/ADCP pathway activation by several therapeutic antibody drugs: ADCC Reporter Bioassay, Core Kit, Promega (ADCC Reporter Bioassays, FcγRIIIa V158 variant [high affinity], catalog numbers G7010 and G7018; Promega Corporation); and FcγRIIa-H (high affinity) ADCP Bioassay, Core Kit, Promega (ADCP FcγRIIa-H Reporter Bioassay, Core Kit, Promega Corporation, catalog number G9995). The assays were performed following the manufacturer's guidelines and are summarized in the Supplemental Information. The ADCC and ADCP induction was expressed as induction ratio (IR), which corresponds to the detected signal versus the 1 U/mL kit calibrator.

In addition, the antigen-coating capacity was evaluated and confirmed by SARS-CoV-2 ELISAs (Alpha Diagnostic Human anti-SARS-CoV-2 S1 IgG ELISA, RV-405200; and Alpha Diagnostic Human anti-SARS-CoV-2 nucleoprotein IgG ELISA, RV-405100) using Corning 96-well Flat Clear Bottom White Polystyrene TC-treated Microplates (Corning reference no. 3903), the 3 SARS-CoV-2 antigens (N, S, and E: Nucleocapsid-His recombinant Protein [reference no. 40588-V08B; Sino Biological Inc., Beijing, China]; SARS-CoV-2 spike protein in LMNG detergent [reference no. 28702; Cube Biotech, Monheim, Germany]; and Recombinant SARS-CoV-2 Envelope Protein [reference no. 32-190021; Abeomics Inc., San Diego CA], respectively), and 3 different sample types (prepandemic IgG within Gamunex C, and IgG ELISA SARS-CoV-2 high positive and negative single-donation plasmas).

HEK293T cells expressing SARS-CoV-2 spike glycoprotein as a transmembrane protein (reference no. P30908; Innoprot) were then used to verify these functionalities (ADCC/ADCP) for SARS-CoV-2 S antigen. A SARS-CoV-2 spike glycoprotein cell line was stably developed transfecting the HEK293T cell line with a SARS-CoV-2 spike glycoprotein expression plasmid (Innoprot, Derio, Basque Country, Spain).

In these experiments, samples were assayed at increasing concentrations to perform a kinetic curve (concentration/response curve) for the dynamic evaluation of ADCC and ADCP functionalities in all sample types. Plasma samples with high, medium, low, and null positivity for COVID-19 infection (determined by anti-SARS-CoV-2 S ELISA methods) were used as comparators.

Table 1. SARS-CoV-2 Infectivity Neutralization by Hyperimmune Immunoglobulin (ID_{50})[a]

Assay	CBIFA	CCLA	PFU	$TCID_{50}$
N	17	14	3	3
ID_{50} (mean ± SD)	483.5 ± 173.5	1075 ± 277.5	4924 ± 2430	1872 ± 610.3

Abbreviations: CBIFA, cell-based immunofluorescence assay; CLA, cytopathic-cytotoxicity luminometry assay; PFU, plaque-forming units; $TCID_{50}$, median tissue culture infectious dose; ID_{50} (as immunoglobulin dilution), half-maximal inhibitory dilution; N, number of batches tested; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SD, standard deviation.

[a]Equivalent concentrations (μg/mL) are as follows: 207 μg/mL (CBIFA); 93 μg/mL (CCLA); 20 μg/mL (PFU); 53 μg/mL ($TCID_{50}$).

Calculations

Neutralization titers were calculated using GraphPad Prism 8 version 8.4.3 nonlinear regression curve fit as ID_{50}. The titers obtained for different batches (n ≥ 3) are expressed as the mean value ± standard deviation.

RESULTS

Severe Acute Respiratory Syndrome Coronavirus 2 Neutralization

All of the methods tested demonstrated neutralization of infectivity by hIG in the 4 SARS-CoV-2 isolates (USA [1], Italy [1], and Spain [2]). The ID_{50} results are shown in Table 1. Differences in ID_{50} are ascribed to differences in the methodologies used reflecting their differential sensitivities. The D614G mutation was present in the isolates from Spain.

Severe Acute Respiratory Syndrome Coronavirus 2 Variants Pseudovirus Neutralization

The neutralization assays with pseudovirus demonstrated the neutralization capability against WT (original Wuhan virus) spike and all variants: D614G, B.1.1.7 United Kingdom (UK), P.1 Brazilian, and B.1.351 South African (Table 2 and Figure 1). The levels of neutralization were very similar for Wuhan D614G and B.1.1.7 spikes and lower for the P1 and B.1.351 spikes, but they still showed consistent neutralization capacity. The negative control (normal IgG IVIG—prepandemic) showed no detectable neutralization.

Antibody-Dependent Cellular Cytotoxicity/Antibody-Dependent Cellular Phagocytosis Induction

Strong ADCC and ADCP IRs by hIG were observed on plates coated with SARS-CoV-2 N antigen (IR of 6 or higher for ADCC, and an IR of 10 or higher for ADCP) at low hIG concentrations (μg IgG/mL) but not with the E and S antigens. Some ADCP induction by the S antigen was observed at higher concentrations of hIG (IR approximately 2 at 5 mg IgG/mL) (Supplementary Information Figures S1 and S2). The ADCC and ADCP induction response for prepandemic plasma and prepandemic IVIG samples (negative controls) were very weak as expected (IR at approximately 1) at any concentration. These results are summarized in Figure 2A and B.

The ADCC induction ratio by HEK293T cells expressing SARS-CoV-2 S glycoprotein was above negative control value for all batches (n = 9; data analyzed at 100 μg/mL) (Figure 3A). High and medium IgG ELISA SARS-CoV-2 antibody-positive single-donation plasmas were also above the negative control value, but the low SARS-CoV-2 positive and negative SARS-CoV-2 plasmas were not above the negative control. Regarding ADCP induction ratio, the 7 hIG batches and the high SARS-CoV-2 positive plasma were above the IR of the negative samples, whereas medium SARS-CoV-2 positive and negative SARS-CoV-2 plasma were not (Figure 3B).

Pooled hIG and high SARS-CoV-2 positive plasma showed ADCC induction by HEK293T cells expressing SARS-CoV-2 S glycoprotein (Figure 4). Activity correlated with increasing concentrations. Maximal induction ratio in kinetics studies was observed at 150 μg/mL. Higher concentrations of hIG interfered with the read-out systems (data not shown). Prepandemic IVIG samples and other plasmas did not show relevant activity.

DISCUSSION

In this study, we report the SARS-CoV-2 neutralization capacity of hIG products for different virus isolates from several regions of the world and for the most relevant SARS-CoV-2 variants.

Table 2. SARS-CoV-2 Variants Infectivity Neutralization by Hyperimmune Immunoglobulin (ID_{50})[a]

Pseudovirus	Wuhan Original	D614G 2nd Wave	B.1.1.7 UK	P.1 Brazil	B.1.351 South Africa
	ID_{50} Values (Reciprocal Dilution) Mean + SD				
N	3	3	3	3	3
Hyperimmune immunoglobulin	3972 ± 1032	4202 ± 1675	2620 ± 136	822 ± 43	597 ± 68
IVIG prepandemic	<300	<300	<300	<300	<300

Abbreviations: ID_{50}, half-maximal inhibitory dilution; IVIG, intravenous immunoglobulins; N, number of batches tested; SARS-CoV-2 severe acute respiratory syndrome coronavirus 2; SD, standard deviation; UK, United Kingdom.

[a]Equivalent concentrations (μg/mL) are as follows: 25 μg/mL (Wuhan Original); 24 μg/mL (D614G 2nd wave); 38 μg/mL (B.1.1.7 UK); 122 μg/mL (P.1 Brazil); 168 μg/mL (B.1.351 South Africa).

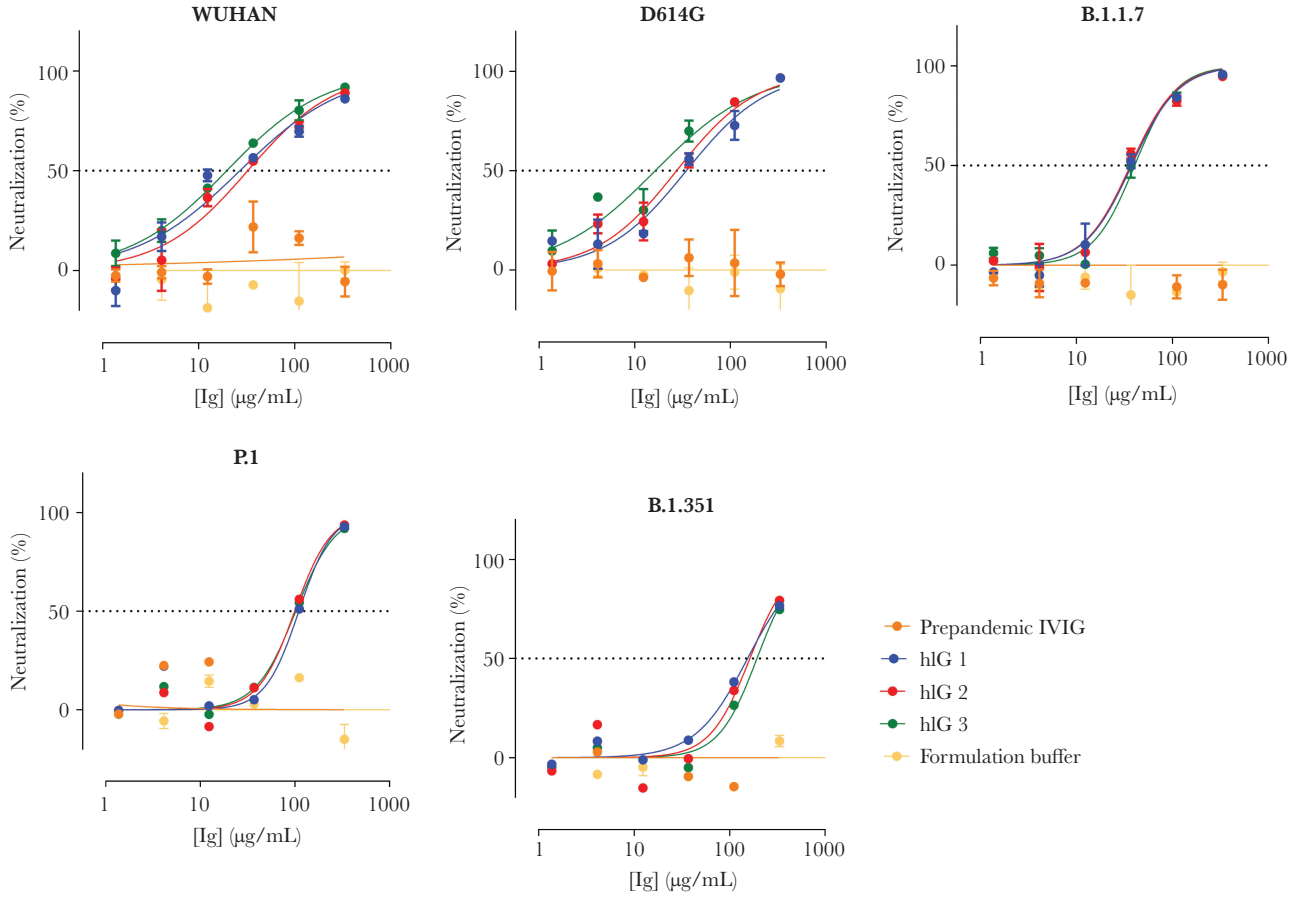

Figure 1. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants infectivity neutralization by hyperimmune immunoglobulin (hIG). Neutralization curves for the indicated SARS-CoV-2 variants (pseudovirus expressing S protein). A prepandemic intravenous immunoglobulin (IVIG) and the formulation buffer were tested in parallel as negative controls. hIG 1, hIG 2, hIG 3 = 3 batches of anti-SARS-CoV-2 hIG.

To our knowledge, this is the first time that several hIG product batches are assayed to evaluate these functionalities. Moreover, the robustness of the anti-SARS-CoV-2 activity of hIG was demonstrated for the first time in different immune effector mechanisms (ADCC, ADCP), with different methodologies, and identifying the viral proteins involved.

Four viral infectivity neutralization methods (CBIFA, CCLA, PFU, $TCID_{50}$) showed strong neutralization of SARS-CoV-2. The ID_{50} values varied because they are method-dependent. Our results using CBIFA were consistent with those reported in the hIG manufacturing characterization (ID_{50} 325 ± 76) [4]. Other neutralization methodologies allowed the detection and reporting of more potent neutralization activity, that is, higher ID_{50}.

Since high neutralization capacity was shown using multiple virus isolates in several virus-cell systems with different methodologies, we can describe the neutralization capacity of hIG as robust and likely to be reproducible under normal physiological conditions after administration to patients. The study of the neutralization capacity with a pseudovirus expressing S glycoproteins from the most relevant SARS-CoV-2 variants (D614G, B.1.1.7, P.1, and B1.351) is especially important given the current situation in the UK, Brazil, and South Africa. In these regions, the predominant variants have been recently classified by the CDC/World Health Organization as variants of great concern [23]. In this study, some reduction of pseudovirus neutralization for P.1 and B1.351 has been shown (preliminary results for some CCP and derived products [24]). Effective neutralization of emergent variants is relevant because the plasma used to produce the hIG was collected before detection of these variants. However, these hyperimmune products were demonstrated to neutralize these new variants consistently, although this capability has not been observed elsewhere [25].

Beyond neutralization, there are other IgG Fc-dependent functionalities of hIG that may play a role in the protection from and/or resolution of SARS-CoV-2 infection, especially when differences in neutralization activity have been detected for some variants [25, 26].

In studies of antigen-dependent Fc function with SARS-CoV-2 antigens, only hIG showed relevant ADCC and ADCP activity for the N protein. This is the most abundant protein in coronaviruses. It is highly conserved and is highly immunogenic

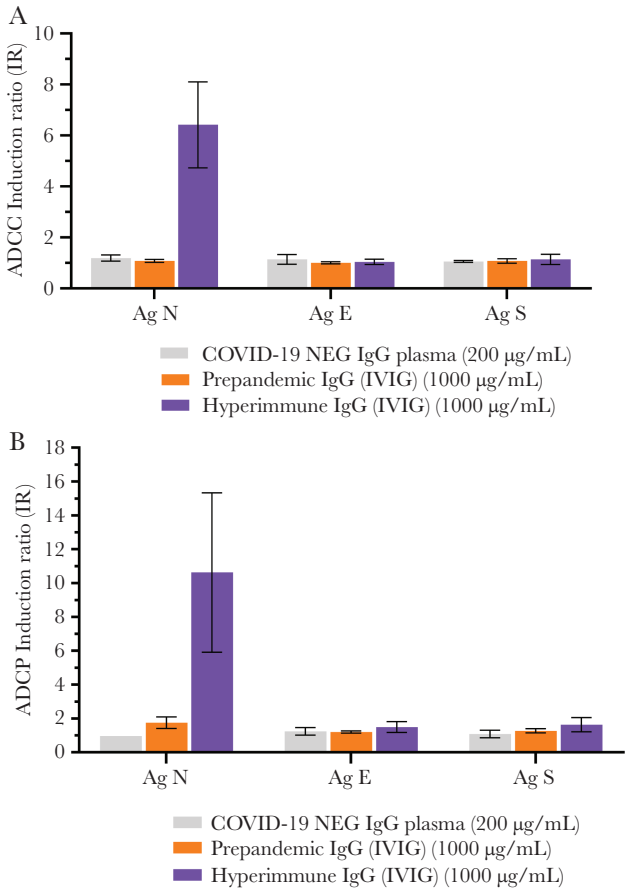

Figure 2. (A) Antibody-dependent cellular cytotoxicity (ADCC) induction ratio with coated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antigens. The 96-well plates were coated with N, E, and S SARS-CoV-2 antigens for the evaluation of ADCC functionality. Single donation plasma samples (SARS-CoV-2 negative [NEG] plasmas) and prepandemic immunoglobulins (IgG) showed no ADCC activity; on the contrary, IgG hyperimmune samples showed marked ADCC activity for the N antigen. (B) Antibody-dependent cellular phagocytosis (ADCP) induction ratio with coated SARS-CoV-2 antigens. The 96-well plates were coated with N, E, and S SARS-CoV-2 antigens for the evaluation of ADCP functionality. Single donation plasma samples (SARS-CoV-2 negative plasmas) and prepandemic immunoglobulins showed no ADCP activity; on the contrary, IgG hyperimmune samples showed marked ADCP activity for the N antigen. COVID-19, coronavirus disease 2019; IVIG, intravenous immunoglobulin.

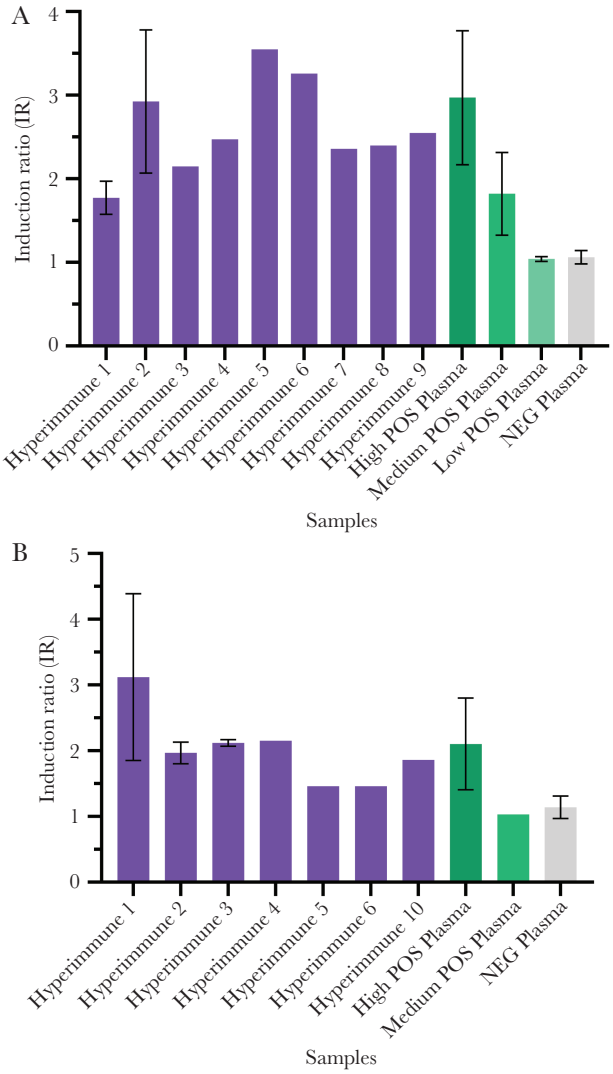

Figure 3. (A) Antibody-dependent cellular cytotoxicity (ADCC) induction ratio with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike glycoprotein expressing HEK293T cells (Innoprot). Nine hyperimmune samples were assayed and showed high ADCC activity (100 µg/mL) when using S expressing HEK293 cells. The SARS-CoV-2 positive (POS) plasma samples (100 µg/mL) also showed this functionality. (B) Antibody-dependent cellular phagocytosis (ADCP) induction ratio with SARS-CoV-2 spike glycoprotein expressing HEK293T cells (Innoprot). Six hyperimmune samples were assayed and showed high ADCC activity (100 µg/mL) when using S expressing HEK293 cells. The SARS-CoV-2 positive plasma samples (100 µg/mL) also showed this functionality. Nonstatistical differences in ADCP induction ratios among hyperimmune batches are attributable to interassay variability. NEG, negative.

[27]. This finding could be particularly relevant for variants capable of escaping anti-S neutralization. In fact, S glycoprotein is one of the most important targets for COVID-19 vaccine and therapeutic research [28].

However, no apparent activity against E and S proteins was observed in Fc function experiments with antigen-coated plates. Although E protein is the smallest of all the structural proteins of SARS-CoV-2, S protein is structurally complex [29]. In both cases, the possibility that the antigen attached to the plate acquired an inadequate conformation to be detected by the test must be considered. S-protein associated ADCC in COVID-19 patients has been recently reported [30]. Although E protein has recently been considered as a potential therapeutic target [31], we further explored Fc functionality related to the more relevant S protein, using HEK293T cells expressing SARS-CoV-2 S glycoprotein.

In HEK293T S cells, all hIG batches induced considerable ADCC and ADCP activity comparable to high titer CCP. This result confirmed that hIG possesses activity against the S protein that was not detected using S antigen-coated plates. Moreover, the ADCC induction ratio was concentration-dependent, with activity at concentrations as low as 25 µg/mL. It is important to remember that the IR value of the positive plasma corresponds

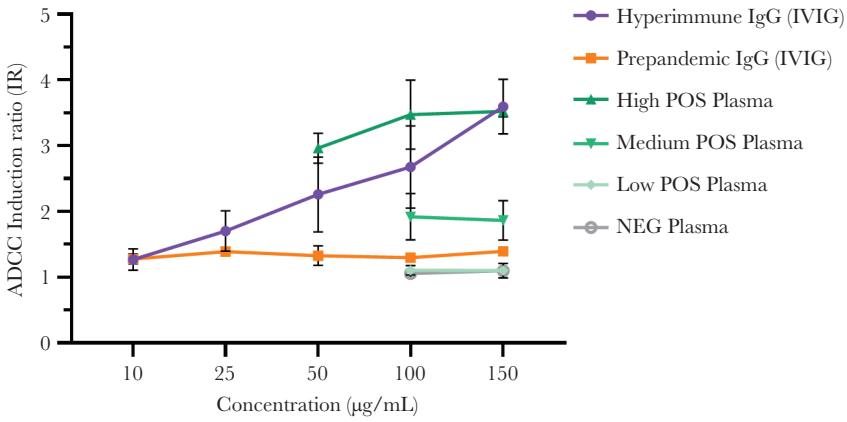

Figure 4. Antibody-dependent cellular cytotoxicity (ADCC) induction ratio kinetic curve in hyperimmune samples, prepandemic immunoglobulins, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) positive (POS) (high, medium, and low SARS-CoV-2 IgG titers) and SARS-CoV-2 IgG negative (NEG) single-donation plasmas, in SARS-CoV-2 spike glycoprotein expressing HEK293T cells (Innoprot). Hyperimmune batches demonstrate high ADCC functionality at incremental concentrations, with a peak at 150 µg/mL. Nonstatistical differences in antibody-dependent cellular phagocytosis induction ratios among hyperimmune batches are attributable to interassay variability.

to a single SARS-CoV-2 positive donor with a high antibody titer, whereas the hyperimmune IR corresponds to multiple donors with variable titers. The ADCC and ADCP are mechanisms for antigen-dependent antibodies through which virus-infected or otherwise diseased cells are targeted for destruction or elimination. This occurs through multiple components of the cell-mediated immune system, primarily through FcγRIIIa expressed on natural killer cells (for ADCC) and by monocytes-macrophages, neutrophils, and dendritic cells via FcγRIIa (CD32a), FcγRI (CD64), and FcγRIIIa (CD16a) for ADCP. The role of antibodies against SARS-CoV-2 N protein in these mechanisms could be a determining factor in resolving SARS-CoV-2 infections through an S-protein independent mechanism. This should be further investigated because, theoretically, protein N is not accessible to antibodies in an intact virus or infected cell.

For other viruses such as influenza A, the effects of nonneutralizing antibodies against internal and more conserved virus proteins suggest that these antibodies play an important role in viral immunity [32]. They reduce virus titers and ameliorate disease via ADCC [33, 34]. Anti-nucleoprotein antibodies can facilitate particle and antigen uptake and presentation, leading to reduced viral titers and morbidity [34–36].

Monoclonal antibody and vaccine efficacy against SARS-CoV-2 are based on anti-S neutralizing activity. In this study, we demonstrated the activity of hIG COVID-19 through other proteins (N) and through mechanisms involving host immune system cells (ADCC and ADCP). This opens the door to combine therapeutic and prophylactic strategies by using these products in combination to increase effectiveness. However, it has been reported that the treatment with CCP has little effect on the outcome of the disease in hospitalized patients [37–40]. Likewise, mAb has limited efficacy once patients are hospitalized, but there is clinical benefit when mAb are administered early in the course of disease [41–44]. Preliminary results suggest that hIG may act in a similar way, that is, by preventing patient hospitalization [45]. In addition, for certain groups of patients such as those with primary or acquired antibody deficiency, the use of SARS-CoV-2 hIG might be much more beneficial compared with typical COVID-19 patients. Nevertheless, direct comparison of different products evaluated with different technologies and/or methodologies is always difficult, if not futile. Precise knowledge of the mechanism of action of hIG will help to predict effectiveness and save time and effort in the selection of the target patient population. In addition, hyperimmune IgG product has antiviral activities beyond neutralization that when combined with neutralization have the potential to provide a more robust treatment against new infectious threats.

CONCLUSIONS

The hIG solutions had strong neutralization capacity against SARS-CoV-2, not only against viruses that plasma donors were exposed to, but also against the new SARS-CoV-2 emerging variants. Under our experimental conditions, viral N and S proteins induced antigen-dependent Fc functions, such as ADCC and ADCP, even at low concentrations. The fact that similar results were obtained with multiple experimental approaches suggests that hIG treatment is a promising therapeutic option for SARS-CoV-2 therapy.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. J. M. D., C. R., and R. G. are full-time employees of Grifols. J. B. and B. C. are founders and shareholders of AlbaJuna Therapeutics, SL. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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