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Gamma-Immunoglobulin Response Characterization, in COVID-19 Convalescent Patients, Against the Spike Protein S2 Subunit with Eight Linear Peptides for Monoclonal Antibody Generation

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ABSTRACT

The SARS-CoV-2 spike protein S2 subunit plays an essential role in the virus-host cell membrane fusion process. Therefore, the subject of this study was to characterize the gamma-immunoglobulin (IgG) response, in a group of COVID-19 convalescent patients, against the S2 subunit with eight linear peptides to generate a monoclonal antibody (mAb) against the immunodominant linear peptide to be used for therapeutic and diagnostic purposes. Results of antibody percentages against assessed linear peptides were 100% for A21P73, A21P74, A21P75, A21P76, M20P51, M20P65, M20P83, and 66.7% for M20P85. Plasma samples were also used for purifying IgG to corroborate specificity against the same linear peptides, where results reproduced those applying plasmas directly to ELISA-plates. Within these peptides, A21P75 was chosen as immunodominant (100% of recognition with higher absorbance). The A21P75 linear peptide showed poor immunogenicity in mice (1:4000–8000 after four doses), allowing the generation of a CB.HS2A21P75 hybridoma for mAb production that recognized the A21P75 linear peptide with middle-to-high affinity constant (K_{aff}) ($0.8\times10^{8}M^{-1}$).

This study concludes that the A21P75 linear peptide is the assessed immunodominant linear peptide for this COVID-19 convalescent patient group. This peptide is located in the HR1 region that plays an important role in the SARS-CoV-2 host cell membrane fusion process and is highly conserved between SARS-CoV-2 and SARS-CoV. Thus, due to CB.S2A21P75 mAb specificity and K_{aff}, it might be the proper reagent to study inhibition of virus-host cell membrane fusion, and as a diagnostic reagent for coronavirus. Finally, the combination of A21P75 linear peptide with other peptides (*e.g.*, receptor binding domain [RBD]) could be suitable reagents for the development of vaccines and therapeutic antibodies with virus infection-blocking capacity.

INTRODUCTION

Coronaviruses (CoV) are a group of related viruses that can cause respiratory tract infection in humans ranging from mild symptoms to the lethal outcome. In December 2019, a new type of CoV emerged in Wuhan, China. The World Health Organization classified this novel virus as SARS-CoV-2 and the disease produced by SARS-CoV-2 infection as Coronavirus disease 2019 (COVID-19). By January 2023, SARS-CoV-2 infected 668,820,532 individuals and resulted in 6,714,775 deaths in 229 countries and territories.[1,2]

SARS-CoV-2 is a single-stranded RNA-enveloped virus with a genome of 29.9 bp in length (GenBank MN908947) encoding 9860 amino acids. The genome consists of ten open reading frames (ORF). In the first ORF (ORF1a/b), about two-thirds of viral RNA is present that encodes for polyproteins 1a and 1b, and 1–16 non-structural proteins such as 3-chymotrypsin-like protease, papain-like protease, and RNA-dependent RNA polymerase. Remaining ORFs encode the structural proteins: spike (S), membrane (M), envelope (E), and nucleocapsid (N); and accessory proteins.[3-5]

The S-protein is responsible for recognizing the host cell receptor (by S1 subunit) and the fusion of virus-host cell membranes (by S2 subunit). This protein consists of an extracellular N-terminal region, a transmembrane domain (TM) anchored to the viral membrane, and a short intracellular C-terminal segment. Once the virus interacts with the host cell, a protein-extensive structural rearrangement occurs, allowing fusion with the host cell membrane.[6]

The total length of S-protein is 1273 amino acids, corresponding with 180–200 kDa (**Figure 1A**). It consists of a signal peptide (SP, amino acids 1–13) located at the N-terminal region, S1 subunit (amino acids 14–685), and S2 subunit (amino acids 686–1273). The S-protein can be cleaved into S1 and S2 subunits by cellular proteases at specific cleavage sites. Then, the S1 subunit performs the function of receptor binding through the receptor

binding domain (RBD, amino acids 319–541).[7,8] Whereas, the S2 subunit is involved in the fusion of membranes to facilitate virus entry into the host cells. It comprises the fusion peptide (FP, amino acids 788–806), heptapeptide repeat sequences (HR1, amino acids 912–984) and HR2 (amino acids 1163–1213), TM domain (amino acids 1213–1237), and a cytoplasm domain (amino acids 1237–1273).[9]

The virus infection process involves multiple steps. First, three independent S-protein cleavage events prime S-protein for interaction with the virus receptor angiotensin converting enzyme 2 (ACE2), allowing subsequent membrane fusion and cell entry. The efficiency in each step contributes to virus virulence and infectivity. Disrupting any one of these steps is the way the host immune system has to combat the SARS-CoV-2 infection[10-12] and thus, is the aim of this research work.

On the other hand, the S2 subunit is highly conserved (**Figure 1B**) and therefore, it may elicit more cross-reactivity compared to the S1 subunit, specifically RBD, which is the least conserved region of the S-protein.[13] Hence, having knowledge of S2 subunit-conserved regions and immunodominant epitopes could help in generating monoclonal antibodies (mAb) for therapeutic applications, vaccine design, and use as diagnostic tools.[14] For instance, vaccines that include epitopes of FP and HR2 domains have induced production of mAbs with the capacity to combat other betacoronaviruses, elicit a stronger- and longer-lasting memory response, and reduce the likelihood of sequence-altering mutations that render the vaccine ineffective.[15,16]

In that sense, researchers have decoded B-cell epitopes within S-protein through a newly constructed 211-peptide microarray system and reported several epitopes within the S2 subunit that may induce antibodies with the capacity to block the SARS-CoV-2 infection mechanism.[17] Besides,

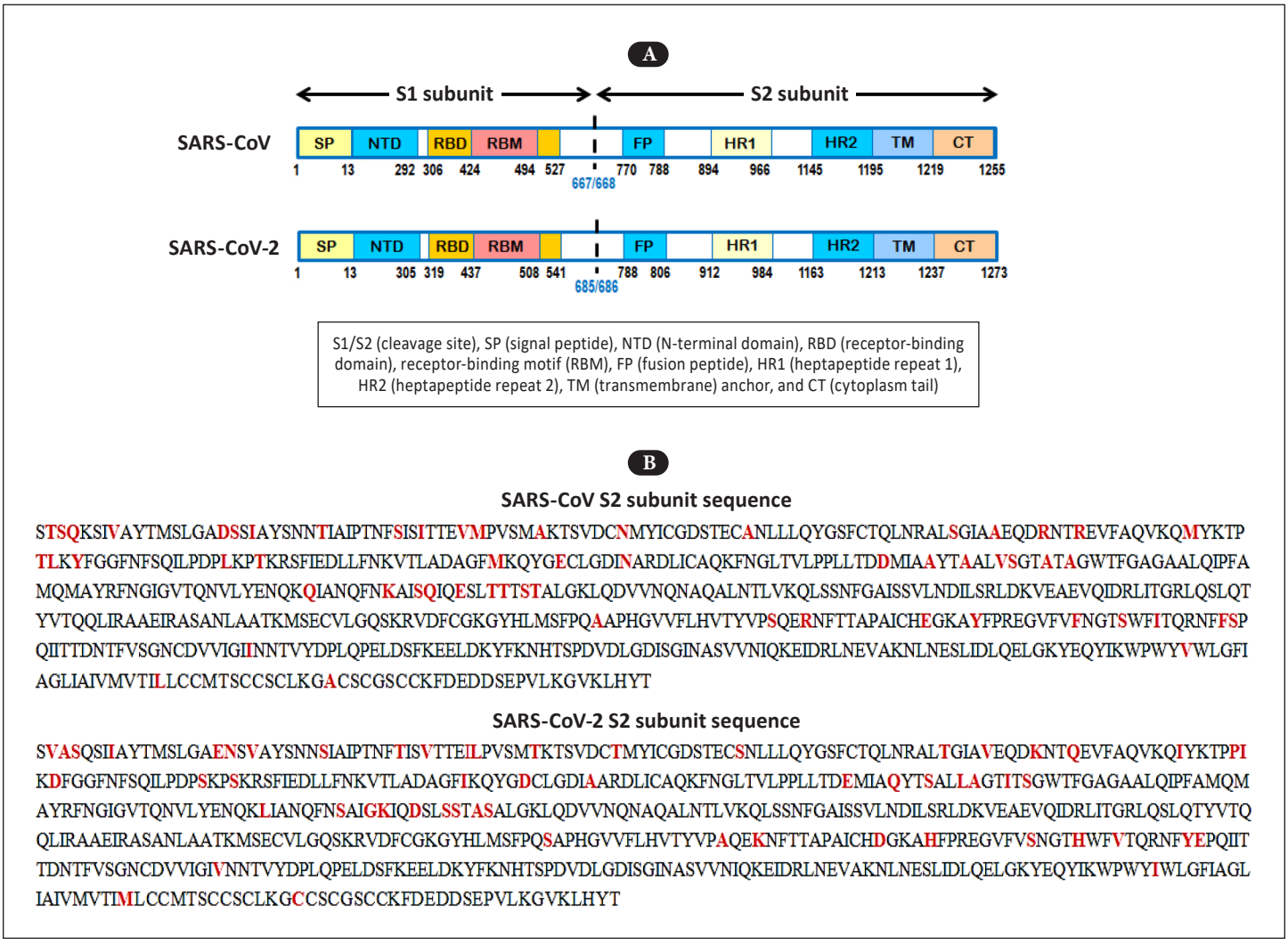

FIGURE 1. (A) Full-length SARS-CoV and SARS-CoV-2 S-protein. **(B)** Sequence identity of SARS-CoV-2 and SARS-CoV S2 subunit. Red amino acids represent no homology between both viruses.

there are two anti-S2 subunit antibodies isolated from COVID-19 patients from which one demonstrated the ability to block SARS-CoV-2 emerging variants.[18]

In another study using an antibody depletion assay against S14P5 (located close to the RBD) and S21P2 (that flanks part of the FP), coupled with a pseudo-typed lentivirus-expressing SARS-CoV-2 S-protein, demonstrated that these antibodies account for a significant fraction (~40%) of the total anti-S-neutralizing response. Conversely, when tested individually, respective epitopes yielded less than a 20% reduction each, substantiating the importance of the S2 subunit. Interestingly, isolated antibodies capable of cross-neutralizing human coronaviruses bound to the conserved stem helix region on the S2 subunit, which revived the hope for developing pan-coronavirus vaccines.[19,20]

Based on this information, the main subject of this study was: (1) the assessment of eight linear peptides (with different degrees of conservation among coronaviruses), located in the S2 subunit, to characterize the gamma-immunoglobulin (IgG) immune response in 22 COVID-19 convalescent patients; and (2) to generate a mAb against the assessed immunodominant linear peptide with the potential for use in therapy and diagnostic applications.

MATERIALS AND METHODS

Linear Peptides

The eight peptides used in this study were produced by the Synthetic Peptide Unit of the Center for Genetic Engineering and Biotechnology (CIGB), Havana, Cuba. The amino acid sequence of each linear peptide was designed by informatic tools (M20P51, M20P65, M20P83, M20P85) or chosen based on literature reports[21-23] (A21P73, A21P74, A21P75, A21P76), as shown in **Table 1**.

Solid-Phase Linear Peptide Synthesis

Peptides were synthesized on a Fmoc-AM/MBHA resin (Merck) by a stepwise, solid-phase procedure using the Fmoc/tBu strategy by Fields and Noble.[24] Fmoc-amino acids were coupled using OxymaPure/DIC (Merck) activation and coupling reactions were monitored by the ninhydrin test.[25] The Fmoc groups were removed with 20% of piperidine in dimethylformamide (DMF). Side chain protector groups releasing and cleaving from the resin were achieved by the treatment with trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIS) (95:2.5:2.5 v/v/v) for 2 h. Subsequently, peptides were precipitated with cold ether, dissolved in 40% acetonitrile/H_2O, and freeze-dried. Crude peptides were purified by a reversed-phase high-performance liquid chromatography (RP-HPLC) and the purity, identity, and molecular masses were confirmed by electrospray ionization mass spectrometry (ESI-MS).[26]

RP-HPLC for Linear Peptide Purification

Peptide purification was performed on an ÄKTA Pure 150 HPLC system (Cytiva Life Sciences). Separations were achieved with a RP-C18 column (30×250 mm, 10 µm, Vydac/VWR). A linear gradient from 10–52% of solvent B (0.05% v/v of TFA in acetonitrile) over 60 min and a flow rate of 50 mL/min were applied. Detections were accomplished at 226 nm. Solvent A, 0.1% (v/v) of TFA in water, was used to equilibrate the column.[27]

RP-HPLC for Linear Peptide Purity Analysis

Analytical separation of peptides was performed in a RP-C18 column (4.6×150 mm, 5 µm, Vydac/VWR). A linear gradient from 5–60% of the same solvent B over 35 min and a flow rate of 0.8 mL/min were applied. Solvent A was used for equilibration. Chromatograms were obtained at 226 nm.[27]

TABLE 1. Information on SARS-CoV-2 S-protein S2 subunit linear peptides used in this study.

Linear Peptides	Regions	Residues	Amino Acid Amounts	Amino Acid Sequences	Homology w/SARS-CoV (%)	Detected in Convalescent Individuals
M20P51	S1/S2	672–691	20	SYQTQTNSPRRARSVASQSI	31.6	1-4-6-7-8-9-10-11-12-16-17
M20P65	FP	788–814	26	KTPPIKDFGGFNFSQILPDPSKPSKR	80.8	6-15-16-17
M20P83	FP/IFP	810–824	15	KPSKRSFIEDLLFNK	99.9	1-2-3-6-7-8-11-12-13-15-16
M20P85	FP	805–824	20	LPDPSKPSKRSFIEDLLFNK	90	1-2-3-6-7-8-9-10-11-12-16-17
A21P73	IFP	901–908	8	MAYRFNGI	100	1-2-3-6-7-8-9-10-12-14-16-17-18
A21P74	HR1/HR2	1133–1140	8	NNTVYDPL	100	1-2-3-4-6-8-9-10-12-15-16-17-18
A21P75	HR1	943–989	47	ALGKLQDVVNQNAQALNTLVKQLSS	100	1-2-3-4-5-6-10-11-12-14-16-17-18
A21P76	HR2	1167–1203	37	DISGINASVVNIQKEIDRLNEVAKNLNESLIDLQELG	100	1-3-4-6-8-10-12-13-14-15-16-17-18

Electrospray Ionization-Mass Spectrometry (ESI-MS) for Linear Peptide Identity Analysis

Peptide analysis was achieved in a hybrid quadrupole time-of-flight (Q-Tof-2) instrument (Waters) fitted with a nanospray ion source. Capillary and cone voltage were set to 900 and 35 V, correspondingly. Data withdrawal and processing were achieved with the MassLynx (version 4) package (Waters).[28]

Plasma Isolation

Plasma samples were obtained from 22 convalescent COVID-19 patients chosen at random from different provinces of the country. Blood was drawn and allowed to clot for 1 h at room temperature before centrifuging for 15 min at 710×*g* in a Hettich Zentrifugen. Subsequently, plasmas were collected and stored at –20°C.

Total Protein Concentration Determination

Purified antibody concentration was determined by the Lowry *et al.* method.[29]

Purity Analysis by SDS-PAGE

Purity of the antibody preparation was estimated by SDS-PAGE following the Laemmli method.[30] CB.Hep-1 mAb was used as an assay control. The mAb was obtained by the standard fusion procedure performed after immunization of a BALB/c mouse with purified rec-HBsAg obtained from plasma of a chronically infected hepatitis B virus (HBV) patient.[31]

Enzyme-Linked Immunosorbent Assay (ELISA) to Define Assay Working Conditions

Nunc MaxiSorp plates (Thermo Fisher Scientific) were coated with eight linear peptides under study (at 10 µg/mL, 45 min at 37°C). Plates were then blocked with skim milk (200 µL/well) and incubated for 45 min at 37°C. Next, 100 µL/well of serum samples from a healthy individual (dilutions 1:10, 1:50, 1:100, 1:500, and 1:1000) were applied to the plates before placing in a humid chamber and incubated again for 1 h at 37°C. After incubation, the plates were washed once more and incubated for 45 min at 37°C with goat antibodies (anti-human IgG) from CIGB, Sancti Spíritus, Cuba (CIGB-SS) conjugated with horseradish peroxidase (HRP, Sigma-Aldrich). Reaction was developed with 100 µL/well of substrate solution (100 µL of 3,3',5,5'-Tetramethylbenzidine [TMB], 20 µL of hydrogen peroxide, and 10 mL of substrate buffer). Reaction was stopped after 10 min with 50 µL of 2 M H_2SO_4 and the absorbance was always determined at 450 nm in a micro ELISA reader (Titertek Multiskan MC340, Labsystems).

ELISA to Determine Specificity of the Convalescent Patient Plasma IgG Against Eight Linear Peptides

For this experiment, the previous protocol was followed using the defined working dilution (1:500). The serum from a healthy individual (not previously infected with SARS-CoV-2) was used as a negative control in the assay. Values greater than the sum (the mean + 3 × the standard deviation) for each peptide were considered as positive. To determine the number of convalescent patients with specific antibodies involved in this analysis, an initial test with plasmas of 50 convalescent patients challenged against the whole RBD molecule was performed. Only 22 were positive.

IgG Purification from Convalescent COVID-19 Patient Plasmas

IgG were purified by Protein A Sepharose Fast Flow matrix (Cytiva Life Sciences) from six convalescent COVID-19 patient plasma samples (those with the largest amounts of assessed linear peptides) using 150 mM PBS, pH 8.0, and 100 mM citric acid, pH 3.0, as adsorption and elution buffers, respectively. Purification runs were performed in PD-10 columns operated at 60 cm/h of linear flow rate. Next, the eluted fraction buffer was changed to 20 mM Tris/150 mM NaCl, pH 7.6, in a 16/30 chromatographic column (Cytiva Life Sciences) operated at 100 cm/h. The final fractions were filtered through Minisart 0.2 µm filters (Sartorius).

Mouse Immunization Procedure

Five six-week-old female BALB/c mice were subcutaneously primed once every two weeks with 50 µg of A21P75 linear peptide. The peptide was emulsified with Complete Freund's Adjuvant (Sigma-Aldrich) for the prime immunization and Incomplete Freund's Adjuvant (Sigma-Aldrich) for three subsequent doses. Three days before splenectomy, one of the immunized mice was sensitized intraperitoneally with 50 µg of the A21P75 linear peptide dissolved in 150 mM PBS, pH 7.2. All animal experiments were performed following the CIGB Animal Care and Use Committee Recommendations (in accordance with international guidelines for the ethical treatment of animals).

Hybridoma Generation and Antibody Production

Hybridoma was generated following protocol described by Köhler and Milstein[32] with some modifications. All clones were screened to determine the presence of antibodies specific for A21P75 linear peptide using the indirect antibody capture ELISA described later. Then, subcloning processes were performed by the limit dilution method.[33] The hybridoma producer of the CB.S2A21P75 mAb specific for the A21P75 linear peptide was finally isolated and used

to produce mAb. Specifically, CB.HS2A21P75 hybridoma cells were seeded into a 25 cm^2 T-flask at 3.0×10^5 cells/mL. Then, culture was expanded to 75 cm^2 T-flasks to produce the cell amount needed to inoculate a 1 L spinner flask. The cell culture was always kept at 37°C in an atmosphere between 5–7% CO_2 using protein-free PFMH-II medium (Gibco, Thermo Fisher Scientific) and replaced every 48 h to reach maximum viable cell density. A MINIFOR stirred-tank bioreactor with biomimicking fish-tail disc (Lambda Laboratory Instruments) was inoculated with hybridoma cells at 4.0×10^5 cells/mL (90% viability) and operated in fed-batch mode for 15 days.[34] All key parameters were automatically controlled.

ELISA for Mouse Serum Titration and Selection of Hybridomas

Nunc MaxiSorp plates were covered with A21P75 linear peptide. Plates were then blocked with skim milk (200 µL/well) and incubated for 45 min at 37°C. Next, samples from mice sera and fusion supernatants were applied to the plates (100 µL/well) for 1 h at 37°C. After this, plates were washed with 150 mM PBS/0.05% Tween-20, pH 7.6, and incubated for 45 min at 37°C with a conjugate of anti-mouse IgG produced in goats (CIGB-SS) with HRP (Sigma-Aldrich). Reaction was developed with 100 µL/well of substrate solution (100 µL TMB, 20 µL hydrogen peroxide, 10 mL substrate buffer). Reaction was stopped after 15 min with 50 µL of 2 M H_2SO_4. The absorbance was always determined at 450 nm in the Titertek micro ELISA reader. Wells were considered positive when absorbances were higher than average absorbance + 3 × the standard deviation of pre-immune serum absorbances.

CB.S2A21P75 mAb Purification

CB.S2A21P75 mAb was purified from the bioreactor supernatant. The supernatant (100 mL) was applied to a Cytiva Protein A Sepharose CL-4B affinity chromatography column (PD-10) using 1.5 M glycine/3 M NaCl, pH 8.9, as adsorption buffer, and 100 mM citric acid (at pH 6.0, 5.0, 3.0) as individual elution buffers. The column was loaded with 10 mL matrix and operated at 30 cm/h for a residence time of 0.19 h. After elution, the pH was then adjusted to neutral with 2 M Tris-HCl at a gentle agitation.

CB.S2A21P75 mAb Affinity Constant Determination

The CB.S2A21P75 mAb affinity constant (K_{aff}) was determined by the non-competitive enzyme immunoassay described by Beatty *et al.*[35] Microtiter plates were coated with the A21P75 linear peptide (5 µg/mL, 2.5 µg/mL, 1.25 µg/mL, 0.65 µg/mL) and incubated with the CB.S2A21P75 linear mAb. Plates were subsequently incubated with a HRP-goat antibody conjugate and the reaction was revealed using o-Phenylenediamine (OPD) as substrate and 0.015% H_2O_2 in citrate buffer, pH 5.0. The reaction was stopped by adding 50 mL of 2 M H_2SO_4. The amount of antibodies adhering to the antigen on the plates was reflected by the enzyme product measured by optical density at 492 nm using the Titertek ELISA reader.

Statistical Analysis

To determine working conditions, results obtained from the ELISA assay were statistically analyzed by the *F*-test to show any significant differences among means of sample dilutions and then the Duncan's multiple range test was applied. The significance level applied in all cases was 95%. The processing and statistical analysis of data was done with the Statgraphics Centurion program (version 15.2, StatPoint Technologies).

RESULTS AND DISCUSSION

An attractive approach to interfering with viral infection and preventing coronavirus disease is to block the virus-host cell membrane fusion process using inhibitory therapeutics (peptides or non-peptides) capable of binding regions involved in virus-host cell interaction or inducing an immune response against these regions.[36] Based on this, the IgG response of 22 convalescent COVID-19 patients against eight linear peptides of the S-protein S2 subunit was assessed to generate mAb specific for the assessed immunodominant linear peptide.

The eight linear peptides assessed in this characterization study were distributed throughout the whole S2 subunit. Briefly, the M20P51 peptide is located at the S1/S2 subunits border and has 31.6% homology to SARS-CoV. The M20P65 and M20P85 peptides are located in different residues of FP and have 80.8% and 90.9% homology with SARS-CoV, respectively. The M20P83 and A21P73 peptides are located in the internal FP. A21P74, A21P75, and A21P76 are located in the HR regions, sharing 100% homology with respect to SARS-CoV (**Table 1**). The FP and these two HR domains on the S2 subunit play a crucial role in the virus-cell membrane fusion process.[37]

To fulfill this objective, an initial ELISA was done to determine the most appropriate plasma dilution for additional testing of samples from 22 chosen COVID-19 convalescent patients. The ELISA was performed with a plasma sample from a healthy person (never infected with SARS-CoV-2) using several dilutions from 1:10 to 1:1000 (**Table 2**). As shown, the statistical analysis revealed significant differences

TABLE 2. Results of plasma assessment at different dilutions following the *F*-test (absorbance at 450 nm) and Duncan's multiple range test (95% of confidence) against eight linear peptides of the S-protein S2 subunit.

Linear Peptides	Plasma Dilution				
	1:10	1:50	1:100	1:500	1:1000
A21P73	0.051	0.045	0.047	0.049	0.049
A21P74	0.059	0.049	0.048	0.053	0.053
A21P75	0.052	0.052	0.047	0.046	0.046
A21P76	0.062	0.061	0.047	0.047	0.047
M20P51	0.057	0.045	0.042	0.042	0.042
M20P65	0.049	0.047	0.054	0.052	0.052
M20P83	0.049	0.047	0.052	0.050	0.050
M20P85	0.050	0.048	0.048	0.045	0.045
	$p=0.0019$				
Mean	0.054	0.049	0.048	0.048	0.048
SD	0.005	0.005	0.004	0.004	0.004
Homogeneous Groups	X	X	X		
			X	X	X

among the means of five dilutions, since the *p*-value of the *F*-test was ≤0.050 ($p=0.0019$). Afterward, the Duncan's multiple range test was applied to determine differences among groups. No differences were seen among absorbances corresponding with 1:10, 1:50, and 1:100 dilutions, as well as among 1:100, 1:500, and 1:1000 dilutions. This meant that any dilution of either group not presenting differences could be chosen to carry out further tests using samples of 22 COVID-19 convalescent patients. Therefore, the plasma dilution chosen for the experiments was 1:500.

The analysis was important because, as the amount of specific antibodies was unknown and there were differences among the 22 COVID-19 patients, further dilution could skew the results. **Figure 2A** shows the percentage of convalesced patient plasma recognizing each linear peptide, which was 100%, with the exception of the M20P85 peptide at 66.7%. As can be seen, there was no immunodominant linear peptide. Therefore, an additional analysis was carried out to select the assessed immunodominant peptide capable of generating the specific mAb. The subsequent ELISA was done with purified (Protein A Sepharose affinity chromatography) IgG antibodies. The idea was to eliminate potential interferences produced by the complex characteristics of the plasma. From this experiment, the highest recognition was directed against the A21P75 linear peptide (**Figure 2B**). Therefore, the A21P75 linear peptide was chosen as the immunodominant linear peptide to generate the specific mAb. Nevertheless, the aim of generating a cocktail of high-affinity mAb specific to all of these immunogenic linear peptides capable of inhibiting the membrane fusion process has not been discarded. This work will be

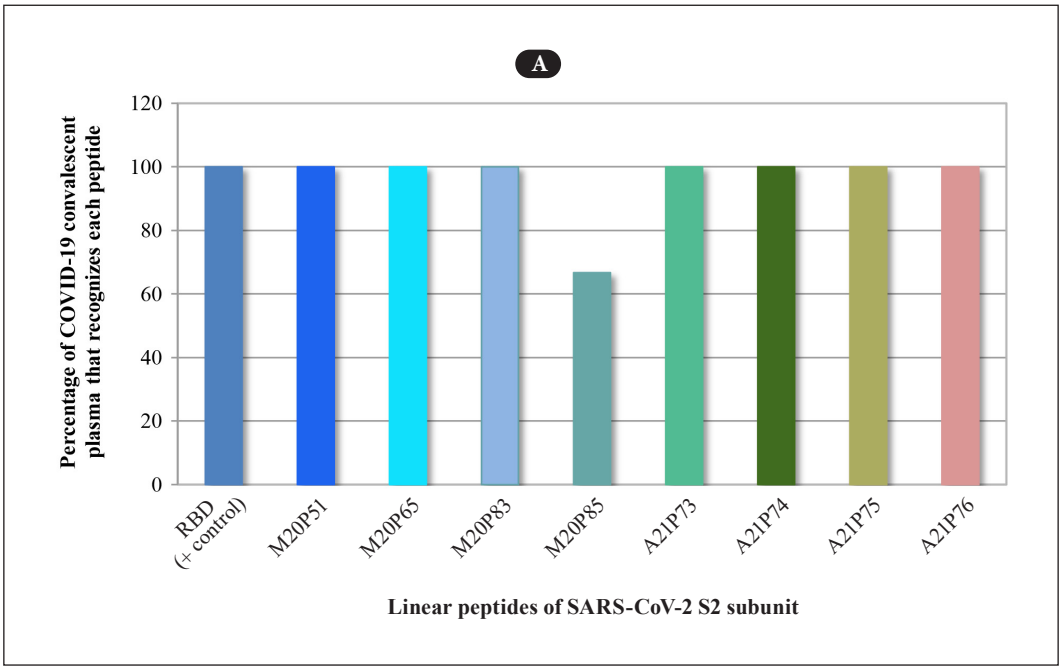

FIGURE 2A. Plasma samples. Recognition of S2 subunit linear peptides by samples from COVID-19 convalescent patients (positive=absorbance of HP + 3× standard deviation).

described in subsequent papers.

Regarding the importance of working with the A21P75 linear peptide, the HR1 and HR2 regions are composed of a repetitive heptapeptide sequence: HPPHCPC.[38] These two regions form the six-helical bundle (6-HB) essential for the viral membrane-cell fusion and virus entry.[9] Coincidentally, other researchers have also demonstrated that peptides derived from HR regions have shown to be highly effective in inhibiting virus entry.[39] Evidently, this issue could justify why the humoral response of COVID-19 convalescent patients is directed toward these regions within the S2 subunit. Therefore, these findings could contribute to not only vaccine development, but also to therapeutics like mAb (single or cocktails), which could effectively reduce the harm caused by escaped mutants.[40] Other examples found in the literature evidenced that peptides derived from the HR1 and HR2 regions of viral fusion glycoproteins can effectively induce an immune response having the ability to inhibit virus infections caused by retroviruses, paramyxoviruses, and other coronaviruses, presumably by interfering with the formation of the 6-HB and inhibiting membrane fusion initiation.[41]

In addition, as has been demonstrated, the genomic characterization of SARS-CoV-2 revealed that this novel betacoronavirus is closely related to SARS-CoV, sharing approximately 79% of the genomic sequence.[13] In this sense, a HR2-specific sequence based on a lipopeptide fusion inhibitor named IPB02, has been shown to be highly potent in blocking S2 subunit-mediated cell fusion for both SARS-CoV and SARS-CoV-2.[42]

Likewise, it has been reported that ferritin nanoparticles conjugated with 70% RBD and 30% HR subunits were able to elicit abundant S-protein-specific neutralizing antibodies in mice.[39] Interestingly, only neutralizing antibodies induced by RBD-HR-region nanoparticles, as opposed to those induced by RBD nanoparticles only, were able to neutralize other coronaviruses. This underlines the importance of the S2 subunit in the search for pan-CoV prophylactic and therapeutic solutions.[39]

From a diagnosis standpoint, the SARS-CoV-2 S2 subunit shows almost 90% sequence homology with SARS-CoV S2 subunit (**Figure 1B**). Within this structurally conserved subunit, the HR regions had 100% of homology.[43] Thus, the use of mAb versus the A21P75 linear peptide would allow the identification of patients with COVID-19 infection but not discriminate among these coronavirus types.

With regard to mAb generation against the A21P75 linear peptide, the process started by immunizing BALB/c mice with the A21P75 linear peptide. After that, mAb-producing hybridoma cells were generated by fusing the A21P75 linear peptide-sensitized antibody-producing splenocytes (B-lymphocytes) with myeloma cells lacking the hypoxanthine-guanine phosphoribosyltransferase (HPRT) enzyme.

As described earlier, each of the five mice were immunized subcutaneously with 50 µg of the A21P75 linear peptide emulsified with complete and incomplete Freund's adjuvant, respectively. Contradictory results of specific antibody titration revealed that the A21P75 linear peptide (under the study

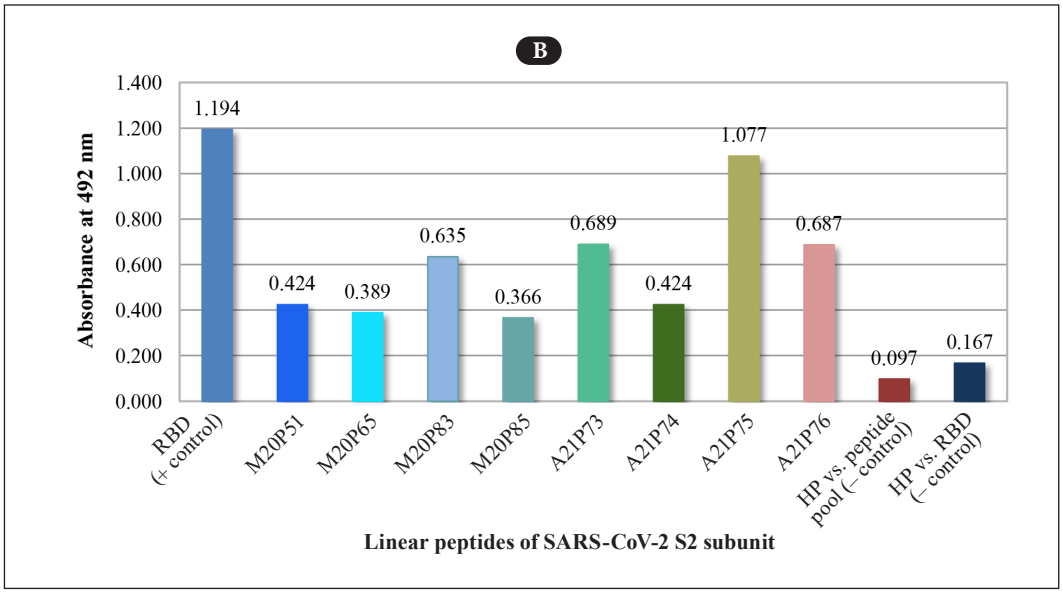

FIGURE 2B. Purified antibodies. Recognition of S2 subunit linear peptides by samples from COVID-19 convalescent patients (positive=absorbance of HP + 3× standard deviation).

conditions used) achieved poor immunogenicity in mice by reaching titers from 1:4000 to 1:8000 after four doses. There are several factors that can affect outcomes. For example, key factors include the preparation of the immunogen (*e.g.*, procedures and adjuvant characteristics), dissimilarities in mouse species, antigen size, and chemical complexity. Any of these properties can be enough as to provoke animals into not recognizing the target substance, or ignore it altogether.

Besides, small compounds like peptides are not complex enough to induce an immune response or to be processed in a manner that elicits the production of specific antibodies. Thus, poor A21P75 linear peptide immunogenicity could be attributed to the peptide size (47 amino acids, 5.03 kDa, as illustrated in **Table 1**). Coincidently, it has been reported that small compounds (<1 kDa), as well as moderately sized molecules (from 1–6 kDa), have not been immunogenic, while compounds with a molecular weight ≥6 kDa can be immunogenic.[44] The explanation lies in the fact that smaller compounds can be bound by the immunoglobulin M (IgM) on the surface of B-lymphocytes, but are still not large enough to facilitate the required crosslinking of mIgM molecules for the receptor-mediated antigen endocytosis.

The solution to this is usually in the conjugation of small antigens with very immunogenic, large carrier proteins.[45] However, in general, it induces a strong immune response specific for carrier proteins and not against the conjugated peptide. Therefore, to create the best immunogen, a recommendation might be to prepare the conjugates with several different carriers and a range of pepide-carrier, protein-coupling ratios, which takes time and resources. Our previous experiences with S-protein peptides conjugated to carrier proteins (KLH and P64k) have required multiple immunizations of subcutaneous doses (>5) to generate relative higher titers of antibodies specific for the target peptides in mice.[46]

Finally, another issue that has to be explored is the immunization route. For instance, vaccines consisting of synthetic peptides representing cytotoxic T-lymphocyte (CTL) epitopes have long been considered a simple and cost-effective approach to treat cancer. However, the efficacy of these vaccines in the clinic, in patients, is still questioned. Peptide vaccines to elicit CTLs in the clinic have routinely been administered in the same manner as vaccines designed to induce antibody responses: injected subcutaneously, and in many instances, using Freund's adjuvant. But, some reports demonstrated peptide vaccination via the unconventional intravenous route of immunization was able to generate substantially higher CTL responses, as compared to conventional subcutaneous injections.[47]

In this study, after the mice were immunized, the initial culture of hybridomas contained the mixture of antibodies derived from different primary B-lymphocyte clones, each secreting a specific antibody into the culture medium. They were screened by means of ELISA plates coated with the A21P75 linear peptide.

Results showed that only six wells had the capacity to recognize the A21P75 linear peptide. The well with the higher absorbance against the A21P75 linear peptide was then separated by limiting dilution and recloned four times to reach 100% of wells with the A21P75 linear peptide recognition capacity. Positive efficiency criteria were: fusion, 1.08%; first cloning, 61.3%; second cloning, 95.7%; third cloning, 97.1%; and fourth cloning, 100%.

Next, purification of the CB.S2A21P75 mAb (>75%) from the supernatant was accomplished by affinity chromatography. The SDS-PAGE results allowed corroborating a typical IgG profile (heavy and light chain) and very high purity of purified molecule (>97%) (**Figure 3**). These results coincide with other published results with cell culture in bioreactors using protein-free media and a purification step incorporating the same Protein A Sepharose CL-4B affinity resin.[29]

Once the specificity and purity of the CB.S2A21P75 mAb (produced in the bioreactor) was demonstrated, a final test for measuring mAb K_{aff} by the A21P75 linear peptide was performed. This parameter might have a crucial influence on the capacity of the CB.S2A21P75 mAb to bind this epitope

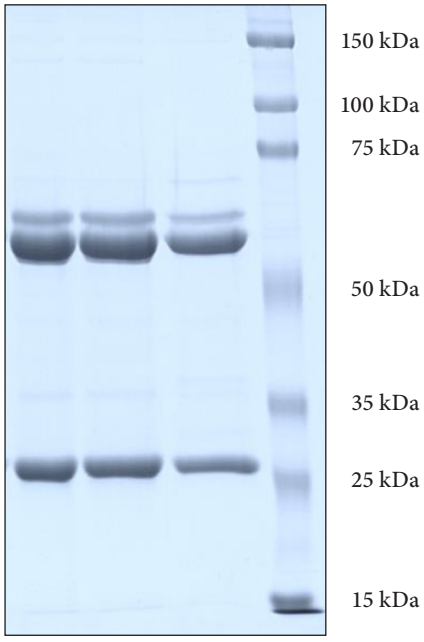

FIGURE 3. SDS-PAGE analysis performed under reducing conditions. **Lane 1:** CB.Hep-1 mAb (control sample); **Lane 2:** CB.S2A21P75 mAb sample; **Lane 3:** human IgG purified from plasma of convalescent patients; and **Lane 4:** molecular weight markers.

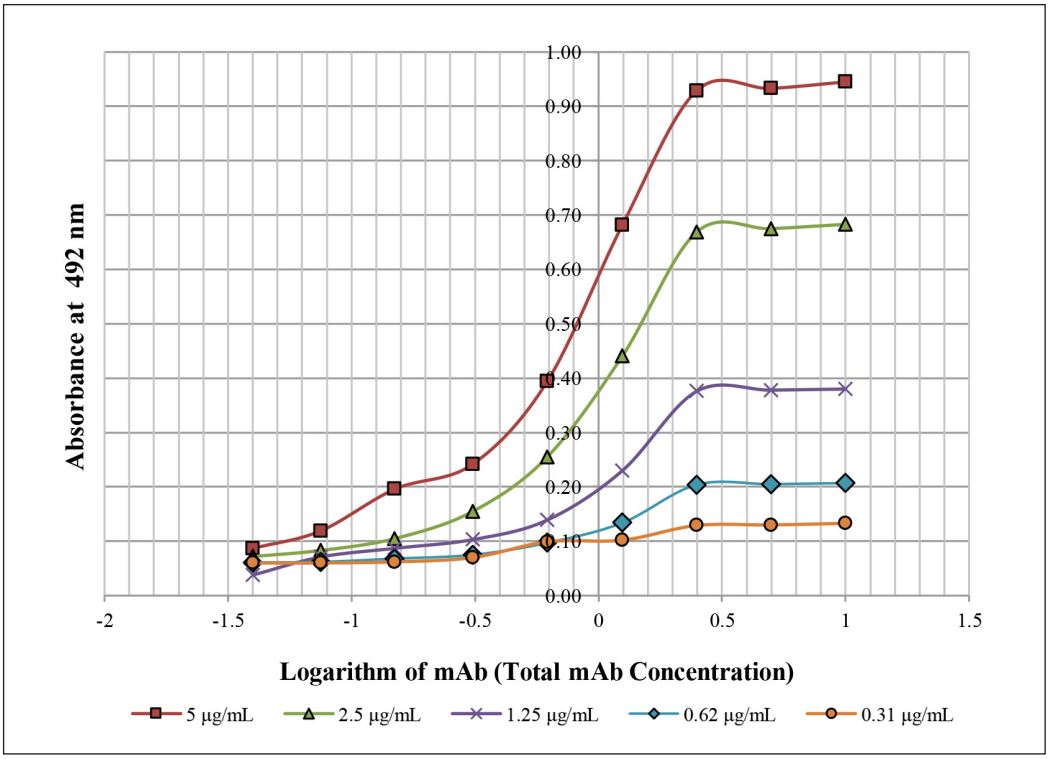

FIGURE 4. ELISA curves performed to determine the K_{aff} of the CB.S2A21P75 mAb.

in the natural conditions of S-protein in the SARS-CoV-2 virus. **Figure 4** illustrates the calculated CB.S2A21P75 mAb K_{aff} corresponded with $0.8\times10^{8}\,M^{-1}$.

Due to fact that most classical immunization procedures involve multiple dosing, the majority of antibodies have K_{aff} values ranging from micromolar (10^{-6} M) to nanomolar (10^{-9} M). High K_{aff} antibodies are considered to be in the low nanomolar range (10^{-9} M) with very high K_{aff} antibodies being in the picomolar range (10^{-12} M). Therefore, the CB.S2A21P75 mAb can be classified as a middle-to-high K_{aff} antibody. Authors speculate that the antigen-binding activity can be enough for the experimental use of this mAb. As an additional note, it is quite probable that the real K_{aff} of this mAb could be even higher since the optimization of the antigen density covering the ELISA plate has been a difficult task when working with the A21P75 linear peptide.

Finally, the combination of the A21P75 linear peptide and other SARS-CoV-2 S-protein S1 subunit peptides (*e.g.*, RBD) and/or the S2 subunit could become the immunodominant linear epitope reagent used for diagnosing COVID-19 infection and for developing vaccines and therapeutic mAbs with virus infection-interfering capacity.

CONCLUSIONS

The assessed immunodominant A21P75 linear peptide is located in the HR1 region and plays an important role in the SARS-CoV-2 cell membrane fusion process. It is highly conserved between SARS-CoV-2 and SARS-CoV. Protein A Sepharose affinity chromatography IgG purification does not allow the elimination of different IgG subclasses, and thus, improves the characterization of polyclonal response from convalescent COVID-19 patient plasmas. The A21P75 linear peptide shows poor immunogenicity in mice, which limits the number of obtainable hybridomas capable of producing mAb specific for this linear peptide. The CB.S2A21P75 mAb recognizes the A21P75 linear peptide with middle-to-high K_{aff}, which could be enough for use in studying this mAb.

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CONFLICTS OF INTEREST

All authors were assessed for conflicts of interest, and none were declared.

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