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Research Article

The Effect of the Anti-Covid-19 Vaccine Abdala Dose Level and Dosing Interval in Rats and Cynomolgus Monkeys

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Abstract

Several anti-Covid-19 vaccines have been developed corresponding to at least 11 vaccine platforms, including novel technology. The Abdala vaccine is based on the RBD fragment of the surface antigen of SARS-CoV-2 expressed in yeast. Previously, we demonstrated the immunogenicity of the recombinant antigen in animals. The present studies examined the impact of Abdala vaccination on the production of RBD-specific antibodies and their functional capacity using two immunization schedules (short 0-14-28 versus long 0-21-42) and two dose levels (10 and 50 µg) in rats and monkeys. The functional capacity of the antibody response was evaluated using an in vitro test to detect blocking antibodies to the ACE-2-RBD interaction and neutralizing activity versus the homologous Wuhan strain. For monkey sera, neutralization against variants of concern (VOC) beta (B.1.351), delta (B.1.617.2) and omicron (BA.1) was also evaluated. Taken together, the findings revealed previously unappreciated differences of the Abdala vaccine in the functional capacity of the antibody response according to the number of inoculations, the dose level, and the dose interval. In this regard, when using the short dose interval, three inoculations seem necessary to improve the functional antiviral response which is also favored with the higher dose level and a long dosing interval. The results in the NHP model also suggest that two doses using a long-time interval (0-21) could improve the efficacy of the Abdala vaccine compared to the short-interval regime (0-14). Cross-neutralization against variants of concern was also demonstrated.

Keywords: Blocking antibodies; COVID-19 vaccines, Monkey; Rats; SARS-CoV-2; Surface antigen

Introduction

According to the World Health Organization vaccine tracker website there are 183 vaccines for Covid-19 under different phases of clinical evaluation [1]. Most of them, approximately one third, are based on the subunit platform aimed at developing neutralizing antibodies targeting the S-protein or its Receptor Binding Domain (RBD) fragment. The RBD interacts with the cellular receptor ACE2 on the surface of susceptible cells to allow viral entry [2]. Therefore, a higher proportion of RBD-specific antibodies neutralize the SARS-CoV-2 virus (severe acute respiratory syndrome coronavirus 2) compared to other non-RBD regions of the S protein [3]. Recombinant RBD (rRBD) polypeptides have been produced in a wide variety of microbial hosts [4-6], such as mammalian cells [7], insect cells [8], and plants [9]. Despite some differences in terms of glycosylation pattern observed between the rRBD expressed in yeast and the natural counterpart of RBD [7], several vaccine candidates based on yeast-derived rRBDs have reached advanced clinical phases showing high levels of efficacy to reduce hospitalizations and severity of the disease [1].

Abdala is a vaccine candidate against Covid-19 based on a rRBD protein (Wuhan-Hu-1 strain) expressed in the *Pichia Pastoris* yeast and adjuvated in aluminum hydroxide. In a previous report, the production and purification process, as well as the physicochemical characterization of the antigen (C-RBD-H6 PP), were described [10]. It is important to note that this antigen differs from others already reported in the literature because it has N- and C-terminal extensions aimed at modulating potential protein-protein interactions and purification purposes, respectively. Although some preliminary evaluation of the immune response for a formulation comprising C-RBD-H6 PP in alum was previously verified in animal models, including rats and monkeys [10], the limitations of the previous report were: 1. Evaluation of experimental lots of the antigen obtained and formulated under laboratory conditions not the pharmaceutical ingredient from the industrial production process or the final vaccine product formulated for human use, 2. Only one immunization regimen (0-14-28) was tested in the NHP model, 3. The immunological results in rats corresponded to a toxicology test of 10 times doses which did not simulate the regime for human use and 4. Lack of experimental evidence on the functional capacity of the antibody response against any SARS-Co-V-2 variants of interest. In the current study, to address previous limitations and better simulate human use of the Abdala vaccine, we evaluated the influence on the kinetic of RBD-specific humoral response of two immunization regimes (short 0-14-28 vs long 0-21-42) and two dose levels (10 and 50 µg) in rats and monkeys. Furthermore, the functional capacity of the antibody response was evaluated using an in vitro test to detect blocking antibodies of the ACE-2-RBD interaction and also the neutralizing capacity of sera to the homologous Wuhan strain in both species. Additionally, neutralizing activity against variants of concern (VOC) beta (B.1.351), delta (B.1.617.2) and omicron (BA.1) was assessed for monkey sera.

Materials and Methods

Composition and Formulation of the Abdala Vaccine and Placebo

The Abdala vaccine was developed and it is produced by the Center for Genetic Engineering and Biotechnology (CIGB, Havana, Cuba). Its pharmaceutical active ingredient is the RBD fragment (amino acids 331-529) of the spike protein of SARS-CoV-2 Wuhan-Hu-1 strain (NCBI Acc. No. YP_009724390) with some N- and C-terminal extensions including a C-terminal six histidine tag for purification purposes. This antigen (C-RBD-H6 PP. Hereinafter referred to as RBDp) was expressed in the *Pichia Pastoris* yeast strain X-33 and obtained as a pyrogen-free product more than 95% pure as described by Limonta-Fernandez and coworkers [10]. The composition of the vaccine is RBDp 0.10 mg/mL, aluminum hydroxide adjuvant (Al^{3+}) (Croda, United Kingdom) 0.60 mg/mL, Na_2HPO_4 0.56 mg/mL, NaH_2PO_4 x 2 H_2O 0.62 mg/mL, NaCl 8.5 mg/mL, Thimerosal 0.05 mg/mL diluted in water. The composition of Placebo groups was the same as the vaccine without the addition of the Active Pharmaceutical Ingredient (API). For monkey immunization batch RPQ021011/0 (CIGB) of Abdala vaccine and batch RPQ21011/0 (CIGB) of Placebo were used. For the rat experimental immunogens were prepared at two concentrations of the RBDp (0.1 and 0.5 mg/mL) in the laboratory following the same procedure and composition under a laminar flow cabinet to ensure sterility and a pyrogen-free product.

Schedules of Immunization

In both species, two schedules of immunization were evaluated by the intramuscular route. There was a short schedule with immunizations on days 0, 14, and 28 and a long one on days 0, 21, and 42. Additionally, in rats, two doses of 10 and 50 µg of the API RBDp of the vaccine were considered. In the case of monkeys, only the highest dose was evaluated. A total of 25 female Sprague Dawley rats (Cenpalab, Cuba) 7-8 weeks old with the weight of 198.6 ± 8.78 g were split randomly into five groups of five animals each according to the dose of the RBDp in the immunogen: 1- Placebo; 2 and 4. RDBp (10 µg); 3 and 5 RBDp (50 µg). In groups 1-3 the short schedule of immunization was used and for the remaining groups, 4 and 5, the long one. The immunogens were prepared with the same composition and procedure of the Abdala vaccine 14-16 h before inoculation and stored at 4ºC under shaking conditions. All animals were immunized with a volume of 0.1 mL per dose by the intramuscular route and bled 12 days after the second dose and 12, 24, 54, and 100 days after the third one (Figure 1).

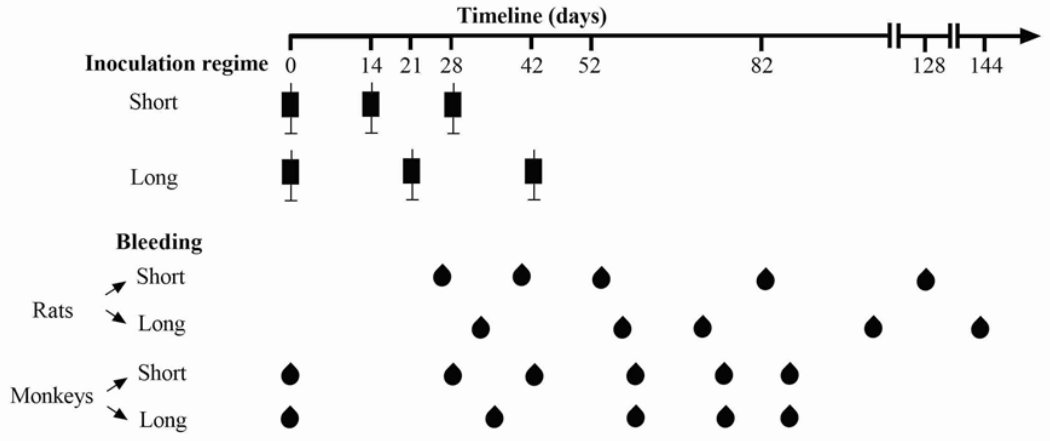

Figure 1: Experimental design. Sprague Dawley rats were randomly allocated to four immunization schedules according to the regime of immunization (short or long) and the dose of RBDp antigen (10 or 50 µg). Additionally, one group of animals received a placebo formulation. *Macaca fascicularis* monkeys were inoculated with a fixed dose of 50 µg of RBDp using either a short or long regime of immunization. Placebo control groups, according to the schedule of inoculation (short and long), were also included. The rats inoculated with the short schedule were bled on days 26, 40, 52, 82, and 128. For the long schedule, on Days 33, 54, 66, 96, and 144. Bleeding times for the monkeys immunized with the short schedule were on days 0, 28, 42, 56, 70, and 84, and on days 0, 35, 56, 70, and 84 for the long schedule.

A total of 8 *Macaca fascicularis* monkeys (Cenpalab, Cuba) more than 2 years-old with weight of 2.812 ± 0.3009 kg were enumerated and allocated at random into 4 groups: 1- Placebo, short schedule of immunization, monkey's number 8951 (herein after # 1); 2- Abdala vaccine, short schedule, monkeys' numbers 11551(2-1), 8961 (2-2), 8989 (2-3); 3- Placebo, long schedule, monkey number 8965 (3); 4- Abdala vaccine, long schedule, monkeys' numbers 8985 (4-1), 8995 (4-2), 11555 (4-3). They were immunized with a volume of 0.5 mL per dose in the deltoid muscle and bled on day 0 and every two weeks after the 2nd and 3rd inoculations (Figure 1). Rats and monkeys used in this study were handled following institutional guidelines, following good animal practices and the experiments were previously approved by the Committee for the Care and Use of Laboratory Animals with code numbers CICUAL 21029 and CICUAL 21018 for the rats and experiments in monkeys, respectively. The welfare of the experimental animals was supervised by a qualified veterinarian or another competent person daily. In the case of monkeys, the body weight and temperature were assessed every 14 days before immunization or blood draw. Furthermore, hematological (i.e. Total Leukocyte Count, Total Erythrocyte Count, Platelet Count, Hemoglobin, Percent Hematocrit, Mean Corpuscular Volume, Mean Corpuscular Hemoglobin and Mean Corpuscular Hemoglobin Concentration) and hemochemical (i.e. Albumin/Globulin Index, Alanine Aminotransferase, Aspartate Aminotransferase, Alkaline Phosphatase, Creatinine, Total Proteins, Albumin, Glucose, Cholesterol, Total Bilirubin, Direct Bilirubin, Triglycerides, Phosphorus, Urea, Calcium, Uric Acid, and Gamma-Glutamyltransferase) parameters were verified to assess the functioning of different organs before the start of the administrations and 20-40 days after the administration of the third dose (end of the inoculation schedule).

SARS-CoV-2 Spike Receptor-Binding Domain (RBD) ELISA

Rats: An in-house indirect ELISA was used to titer the RBD-specific IgG response in serum. High binding capacity 96-well plates (Costar, USA) were coated with RBDp at 2.5 µg/ml in 100 µL of coating buffer (11 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) and incubated overnight at 4°C. Plates were blocked with 2% skim milk in PBS for 1 h at 37 °C. Subsequently, they were incubated with serum samples diluted in 0.2% skim milk, and 0.05% Tween 20 in PBS for 2 h at 37°C. Goat anti-rat IgG (whole molecule) peroxidase conjugate (Sigma-Aldrich, USA) at 1: 5 000 dilution was incubated for 1 h at 37°C. The reactions were then developed with substrate solution (52 mM Na_2HPO_4, 25 mM sodium citrate, 1 mg/ml o-phenylenediamine, and 0.1% H_2O_2) for 10 min at room temperature. The reaction was stopped with 50 µL of 3 M H_2SO_4. Last, the plates were read at 492 nm (A492) in a microplate reader (PR621; Immunoassay Center, Cuba). Washes (at least five) with 0.05% Tween 20 in distilled water were carried out between each step. The threshold value for seroconversion was considered more than 4 times the average of titers in the Placebo group. Sera titers were calculated as the antilog of the resulting value after interpolation of the decimal logarithm of the absorbance values at a fixed serum dilution into a log-log linear regression analysis plotting dilution versus A492nm of the standard curve of

a known titer serum. The titer of the standard curve was defined as the highest dilution that gave more than twice the absorbance of the negative control serum diluted 1:100. Total IgG titers were expressed in Standard Units (STD Units) and reported as the Geometric Mean (GMT) plus 95% Confidence Interval (CI) of the individual sera.

Monkeys: To determine the specific recognition of RBD by the serum of immunized animals, the UMELISA SARS-CoV-2 anti-RBD kit manufactured by the Immunoassay Center (CIE, Havana, Cuba) was used. Briefly, the technique employs ultramicro ELISA plates coated with the RBD fragment of the protein S of the virus. The serum sample was added to the wells. At the same time, a standard curve and negative controls for the assay were added to quantify the antibody titers present in each sample. The addition of a second biotinylated anti-human IgG antibody (cross-reactive with monkeys) allows this isotype to be selected, which in a subsequent step binds to the streptavidin/alkaline phosphatase conjugate. The presence of antibodies was detected through the fluorescent signal indicating the conversion of the fluorogenic substrate 4-methylumbelliferyl phosphate. The fluorescence values of the serum samples of unknown concentration were interpolated in a graph of fluorescence vs concentration of anti-RBD IgG corresponding to the Standard Curve. Results were expressed in Binding Antibody Units per mL (BAU/mL). Values above a threshold of 18 BAU/mL were considered positive.

ELISA-Based Surrogate Virus Neutralization Test

To evaluate the quality of the antibody response generated by immunization, it was determined to what extent the sera of the immunized animals inhibit the binding of RBD to its ACE2 receptor. In this assay [11], flat bottom 96-well ELISA plates (Costar 3590) were coated in Carbonate-Bicarbonate buffer pH 9.6 with 5 µg of the recombinant protein ACE2 coupled to the Fc portion of a murine immunoglobulin (ACE2 mFc) and incubated for 16-20 h at 4ºC. Subsequently, the plates were washed with 0.1% Tween 20 (v/v) in H_2O and blocked with 2% (m/v) skim milk in PBS 1X 0.05% Tween 20 (v/v) for 1h at 37°C. During the same time, serial dilutions (1:20-1:163 840) of the monkey sera or 1:100 and 1:400 dilutions of the rat sera corresponding to the blood extraction time-points were preincubated in U-bottom culture plates (Costar 3799) with 1:25 000 of the RBD protein coupled to the Fc portion of a human immunoglobulin conjugated to peroxidase enzyme (RBD hFc-HRPO). The serum and RBD hFc-HRPO reagent were diluted in 0.2% (m/v) skim milk, 0.05% Tween 20 (v/v) in PBS 1X. ELISA plates were washed 3 times and 50 uL of the preincubation mixture of the serum was transferred to the coated plates. After 90 minutes at 37°C, the plates were washed under the same conditions already described and the substrate Tetramethyl Benzidine (TMB) was added. The reaction was stopped with 2.5 N sulfuric acid after 10 minutes of incubation. Finally, the absorbance at 450 nm was detected in the PR621 plate reader (CIE, Cuba).

With the data obtained from the reading, the percentage of inhibition (%Inhib) at specific dilutions was calculated concerning the maximum binding (Umax: Absorbance obtained from the binding of the RBD hFc-HRPO to the ACE2 fixed to the plate in the absence of serum) after subtraction of absorbance values of the background control wells incubated with all the reagents except serum and RBD conjugate. When a negative value arose it was processed and represented in graphics as zero. The dilution of the monkey's serum that promoted a 50% inhibition (IC50) was calculated with the statistical and curve-fitting package Graph Pad Prism version 8.0.2 ((GraphPad Software, Boston, MA). To accomplish that, experimental curves were expressed as log (serum dilution) vs % inhibition and analyzed with the 4-parameter logistic model (4PL) of variable slope using the least squares fit. If the IC50 value could not be determined because no inhibitory effect was observed at any of the dilutions tested, an IC50=10 was assigned corresponding to a dilution twice as concentrated as the first point of the curves (1:20).

SARS-CoV-2 Microneutralization Assay (MNA)

In this assay, 96-well culture plates seeded with $2x10^4$ cells/well of the VERO E6 cell line, which expresses the ACE2 receptor, were used. Cells were incubated for 24 h at 37°C and 5% CO_2 to form a monolayer grown to 85-90% confluency. Subsequently, the previously inactivated serum at 56°C for 30 minutes was serially diluted 1:2 in a minimal essential medium (MEM, Gibco, UK) with 2% (v/v) bovine fetal serum (SFB, Capricorn, Germany). Each dilution was incubated for 1 h at 37°C with 100 TCID50 (Tissue Culture Infectious Dose) of the Cuban isolates of SARS-CoV-2 D614G variant (CUT2010-2025/Cuba/2020 and 30654/21), beta B.1.351 (34959/21), delta B.1.617.2 (57383/21) and omicron BA.1 (8649/22). Viral suspensions in the presence and absence of experimental serum were used to infect cell monolayers for 96 h at 37ºC and 5% CO_2. After this time, the presence of the cytopathic effect (viral plaques) was verified with the help of an optical microscope and staining with neutral red. The excess vital dye was removed by three washes, and the neutral red incorporated into the cells was dissolved by incubating for 15 minutes at 25°C with a lysis solution composed of 50% ethanol and 1% acetic acid. The absorbance was measured at 540 nm, and the neutralizing titer was reported as the highest dilution of the serum with an absorbance greater than the cut-off value of the assay. The cut-off value was defined as half of the average absorbance values of the wells without the virus.

Statistical Methods

GraphPad Prism software v8.0.2 (GraphPad Software, Boston, MA) was used for all descriptive statistics and Figure representations. Inferential statistical analyses were not performed due to the small number of animals in the studies.

Results

Immune Response in Sprague Dawley Rats

Kinetic of the RBD-specific IgG titers in serum: Four groups of five rats were immunized by the intramuscular route with 10 or 50 µg of alum-formulated RBDp, while five control animals received Placebo only. Furthermore, two immunization regimes (short and long) were compared to study the role of timing between shots in the kinetics of the humoral response. As shown in Figure 2, 12 days after the second shot, there was 100% seroconversion in groups of rats immunized with RBDp at 50 µg with the short or long regimen. In contrast, only 60% (3 out of 5) and 80% (4/5) of rats seroconverted with RBDp at 10 µg using a short or long regimen, respectively. Furthermore, the dispersion of values was also less pronounced in the high-dose groups. Twelve days after the third shot, a booster effect was observed and all groups immunized with RBDp reached 100% seroconversion. This rate of seroconversion was maintained until 100 days of follow-up. However, it appears that a slow decline in IgG titer levels occurred since day 52 (24 and 10 days after the last inoculation for the short and long regime, respectively) (Figure 2). However, GMTs were still high.

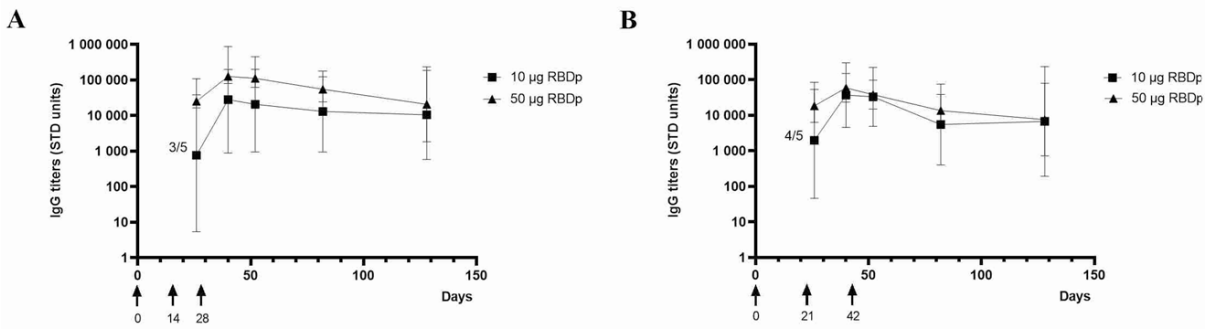

Figure 2: Time course of IgG response to RBD (10 and 50 µg) in rats after a short (A) and long (B) regime of immunization. The ratio of seroconversion is displayed at time points when 100% seroconversion was not attained. Data are expressed as log values and represent the GMT ± 95% CI. STD, standard.

Functionality of the Antibody Response: To analyze the functional capacity of these anti-RBDp antibodies, we evaluated whether they were able to inhibit the interaction with the ACE2 molecule (i.e., the receptor of SARS-CoV-2). To achieve that, an ELISA-based assay was used in which an RBD produced in mammalian cells interacts with ACE2 in the presence of individual sera at fixed dilutions. As shown in Figure 3A, after two shots, the sera of the animals did not show inhibitory activity at 1:100 dilution independently of the dose of RBDp and immunization regime used, except in one rat in the 50 µg group using the short regime. On the contrary, 24 days after the third shot, all groups reached their highest responses, resulting in more than 50% inhibition in most animals except in the group of 50 µg RBDp long regime. The groups that received 50 µg RBDp in the short regimen and 10 µg RBDp in the long regime achieved the highest frequency of response above 50% inhibition, and it was sustained until 100 days after completion of the immunization regime in both groups. The other groups showed some trend of decreasing inhibitory activity with time. Next, we re-evaluated all sera with more than 50% inhibitory activity at a 1:400 dilution to rule out any effect of the antibody concentration in the samples. The results showed a trend toward a decrease in the level of inhibition of the RBD-ACE2 interaction over time (Figure 3B). In fact, 24 days after the third inoculation, most of the groups had an average close to 50% of inhibition, and in the following weeks all the groups were below that level. In view of the previous results, we decided to test the neutralizing activity against the D614G variant of the SARS-CoV-2 virus using a pool of selected sera per group that had more than 50% inhibition at a dilution of 1:100. Therefore, two time points were considered, 24 and 54 days after the third dose. Table 1 showed that all experimental groups developed neutralizing antibodies that lasted at least 54 days at a steady level. As expected, the Placebo group did not develop any positive response.

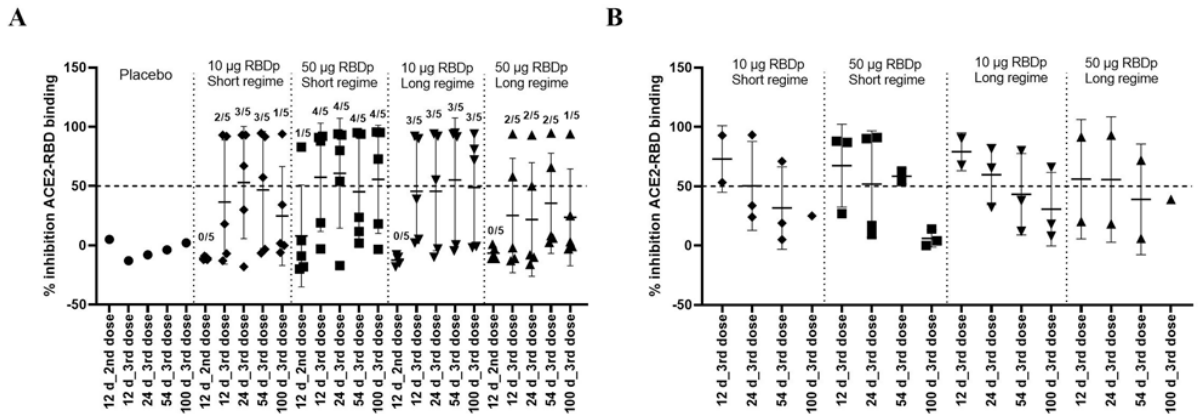

Figure 3: Surrogate Virus Neutralization Test (sVNT) based on antibody-mediated blocking of the ACE2-RBD interaction. The data represent the individual sera of rats diluted 1:100 (A) or 1:400 (B). Animals were immunized with 10 or 50 µg RBD using a short or long regime of inoculation. The average ± SD is also shown.

Groups	Time point (days) after the last immunization (3rd dose)	Neutralization titer
Placebo	24	0
	54	0
10 µg RBDp short	24	1:320
	54	1:112
50 µg RBDp short	24	1:224
	54	1:320
10 µg RBDp long	24	1:160
	54	1:160
50 µg RBDp long	24	ND
	54	1:320

ND: Not done

Table 1: SARS-CoV-2 microneutralization assay (MNA) using a pool of sera obtained from the groups of rats tested at different time points to assess their neutralizing capacity versus the SARS-CoV-2 original Wuhan strain of the virus.

Immune Response in Cynomolgus Monkeys

Kinetic of the RBD-Specific IgG Titers in Serum: Previous studies in rats administering the RBDp antigen intramuscularly in formulations similar to the Abdala vaccine showed its high immunogenicity and induction of high anti-RBD antibody titers with functional capacity. Next, we investigated the elicited immune response using similar immunization schedules in cynomolgus monkeys, an experimental animal species closer to humans. To ensure a better simulation of a prospective human vaccination, animals were immunized with a released vaccine or placebo batch via the same intramuscular route using a short and long schedule of inoculation at a fixed dose of 50 µg of protein. To rule out any major side effects of vaccination, the clinical status of the animals was verified before and after inoculation. The physical appearance and behavior of the primates were observed daily, and no incidents were reported that would indicate any impairment of their physiology or health status. The hematological and hemochemical variables determined prior to the start of the immunizations and after the third administration were within the normal range of these parameters for the specie *Macaca fascicularis* [12] (data not shown). Figure 4A and B represent the kinetics of the anti-RBD IgG response during the 84 days

of the protocol in animals under a short and long schedule of immunization, respectively. On day 0, all animals in the placebo or experimental groups were negative (<18 BAU/mL). Two weeks after the second dose, titers increased in both schedules, with a more homogeneous and higher response in animals receiving Abdala in the long schedule. Nevertheless, under the short regime, there was still one negative animal. Two weeks after three doses, all animals, independent of the immunization regime, achieved the highest titer value and GMTs and range of lower-upper 95% CI (1035 (241.5-1666) short; 634.2 (70.78-15144) long regime) overlapped between the groups. Thereafter, a slight decrease was observed until day 84 of the study. It is important to note that at the end of the protocol, all animals that received Abdala showed titers between 20 and 1000 times higher than those detected in their pre-immune serum.

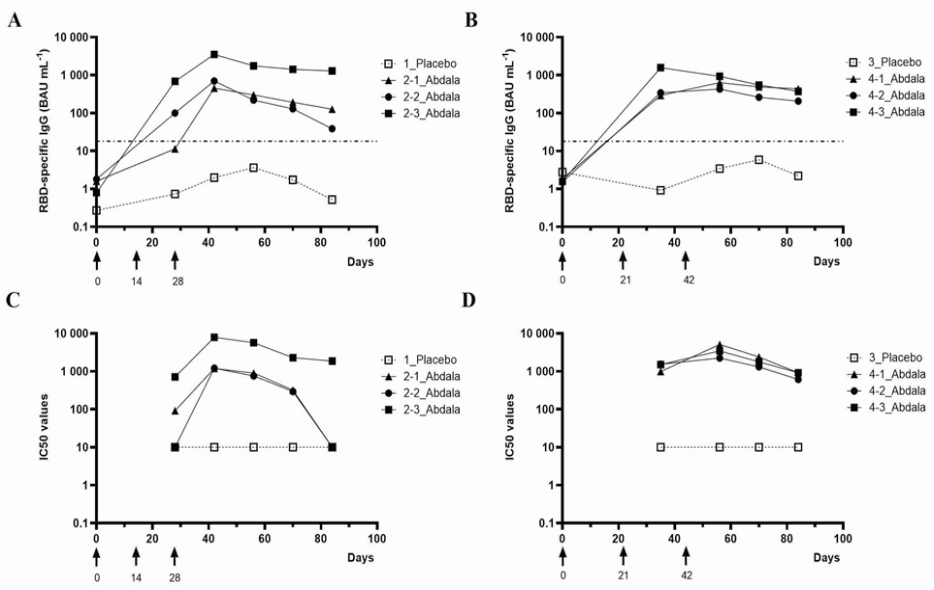

Figure 4: Time course of IgG response to RBD (A and B) and IC50 values of inhibition of RBD-ACE2 interaction (C and D) in sera of monkeys inoculated by the i.m. route with Abdala vaccine using short (A and C) and long (B and D) immunization regimes. Data from individual animals are expressed as log values. The dashed lines indicate a threshold value of 18 BAU/mL. BAU, binding antibody units. IC50, 50% inhibition of the interaction between RBD and the SARS-CoV-2 receptor ACE-2.

Functionality of The Antibody Response: To analyze the functional capacity of such anti-RBDp antibodies, we evaluated whether they could inhibit the interaction RBD-ACE2. In this case, we calculated the IC50 for each serum sample. As shown in Figure 4C and D, in both schedules of immunization, the short and the long ones, the IC50 values peaked 14 days after the third dose, and the mean values were very similar at 3435 and 3583, respectively. However, in the long schedule, the sera of the three monkeys achieved similar levels at all time points compared to the results of the short ones, which were rather dispersed. It is important to note that the ratio of mean IC50 values 14 days after the second inoculation was approximately five times higher in the long versus short schedule (1346 vs 270, respectively). Interestingly, IC50 values dropped at a steady pace of 0.71 and 0.51 times 14 days after the peak (day 28 after the third dose) in the short and long schedules, respectively. A similar further 0.4 times drop 28 days after the peak (day 42 after the third dose) was observed in both schedules. Nevertheless, it is important to highlight that two out of the three monkeys in the short schedule showed no functional response at day 84, which corresponds to 56 days after the third inoculation. To further support the functional capacity of the antibody response, sera from individual monkeys were tested using a classical neutralization assay against the SARS-CoV-2 D614G variant, which comprises the RBD sequence included in the vaccine. Table 2 shows the results of neutralizing titers until day 84 for the short and long inoculation regimens. We also calculated the Neutralization Potency Index (NPI) to assess the relative contribution of IgG response to the neutralization activity of the sera. In this regard, we did not observe an obvious discrepancy in the values when comparing the short and long immunization regimes. Finally, to verify whether such neutralizing responses could cross-neutralize VOC, sera corresponding to 14 days after the third shot were pooled and tested against beta (B.1.351), delta (B.1.617.2), and omicron (BA.1) isolates. As shown in Table 3, Abdala vaccination elicited cross-neutralizing antibodies against these VOC. Nevertheless, the results indicated a similar neutralization capacity of the pooled sera for the delta VOC as compared to the D614G variant, and a lower than 50% Neutralization Potency Index (NBI) for the beta and omicron BA.1 VOC.

Inoculation regime	Group	Monkey ID	Day 0		Day 28		Day 35		Day 42		Day 56		Day 70		Day 84	
			NT	*NPI*	NT	*NPI*	NT	*NPI*	NT	*NPI*	NT	*NPI*	NT	*NPI*	NT	*NPI*
Short Schedule 0, 14, 28	Placebo	1	‹ 20	*0,0*	‹ 20	*0,0*	nd	*nd*	‹ 20	*0,0*	‹ 20	*0,0*	‹ 20	*0,0*	‹ 20	*0,0*
	Abdala	2-1	‹ 20	*0,0*	40	*3,5*	nd	*nd*	447	*1,0*	447	*1,5*	224	*1,2*	80	*0,6*
		2-2	‹ 20	*0,0*	‹ 20	*0,0*	nd	*nd*	224	*0,3*	80	*0,4*	40	*0,3*	80	*2,1*
		2-3	‹ 20	*0,0*	56	*0,1*	nd	*nd*	1280	*0,4*	320	*0,2*	224	*0,2*	224	*0,2*
Long Schedule 0, 21, 42	Placebo	3	‹ 20	*0,0*	nd	nd	‹ 20	*0,0*	nd	nd	‹ 20	*0,0*	‹ 20	*0,0*	‹ 20	*0,0*
	Abdala	4-1	‹ 20	*0,0*	nd	nd	160	*0,5*	nd	nd	881	*1,4*	640	*1,3*	320	*0,7*
		4-2	‹ 20	*0,0*	nd	nd	224	*0,7*	nd	nd	320	*0,7*	320	*1,2*	160	*0,8*
		4-3	‹ 20	*0,0*	nd	nd	112	*0,1*	nd	nd	224	*0,2*	224	*0,4*	112	*0,3*

NT: neutralization titer; NPI: Neutralization Potency Index (NT/RBDp-specific IgG titer)

Table 2: SARS-CoV-2 microneutralization assay (MNA) using individual sera of cynomolgus monkeys at different time points. Neutralization titers were assessed versus the D614G isolate (CUT2010-2025/Cuba/2020).

Inoculation regime	Groups	Neutralizing titers $^{-1}$ (NBI)			
		D614G variant	Beta variant	Delta variant	Omicron BA.1
Short Schedule 0, 14, 28	Placebo	< 10	< 10	< 10	< 10
	Abdala	160 (100%)	60 (38%)	120 (75%)	80 (50%)
Long Schedule 0, 21, 42	Placebo	< 10	< 10	< 10	5
	Abdala	320 (100%)	40 (13%)	160 (50%)	60 (19%)

NBI: Neutralization Breath Index (VOC neutralization titer / D614G neutralization titer)

Table 3: SARS-CoV-2 microneutralization assay (MNA) versus variants of concern (VOC) using a pool of sera from cynomolgus monkeys obtained two weeks after the third dose. D614G isolate code 30654/21, beta B.1.351 code 34959/21), delta B.1.617.2 code 57383/21, and omicron BA.1 code 8649/22.

Discussion

In this report, we show that the recombinant RBD antigen produced in *Pichia Pastoris* and formulated in the Abdala vaccine is highly immunogenic and induces a functional response in a rodent and NHP model. In rats, there was a booster effect on the RBD-specific IgG response with the number of inoculations; however, a functional response in terms of inhibition of the ACE2-RBD interaction and neutralization against the D614G variant of SARS-CoV-2 was only observed after the third dose using either a low (10 µg) or high (50 µg) dose in the short (0-14-28) or long (0-21-42) regimen. This suggests that some level of maturation in the affinity of the antibodies must occur to achieve neutralizing activity. Previous results were replicated after evaluation of a batch of the Abdala vaccine in Cynomolgus monkeys at a fixed dose of 50 µg using the short and long regime of inoculation. In this NHP model, it was also demonstrated the neutralizing capacity of 100% of monkeys' serum against the D614G variant of SARS-CoV-2 after the third and second inoculations in the short and long regimens, respectively. As expected, there was strong agreement between the IC50 values of the ACE-2-RBD inhibition assay and serum neutralizing titers at all time intervals. Previous results also suggest a strong influence of the administration regimen on the functional antiviral response.

At present, limited data are available on the optimal dosing interval for SARS-CoV-2 vaccines. Supportive data on the impact of the immunization regime emerged from observations of higher immunogenicity and efficacy of the Oxford/AstraZeneca ChAdOx1 NCoV-19 Vaccine after longer periods of time between the primary and secondary inoculations [13]. Recently, similar results have been reported for the use of mRNA vaccines in the elderly [14,15] and young individuals [16] suggesting that extended schedules of immunization can promote higher immunogenicity and efficacy of vaccines independently of the vaccine platform. However, it is important to consider that under the pressure of an epidemic, a short immunization schedule or regime would shorten the vaccination time and accelerate the induction of protective immunity in the population. It is worth noting that results in rats and monkeys suggest that further booster shots might be necessary to sustain the level of antibody and functional response after a couple of months because they have a trend to decrease.

Although several reports have previously shown that recombinant RBDs produced in yeast can be used to produce an effective subunit vaccine against COVID-19 [5,17,18], the Abdala vaccine is one of the few products that have already reached phase 3 efficacy trials [1] and received Emergency Use Authorization. The efficacy of the vaccine against symptomatic

COVID-19 at a dose of 50 µg using the regimen of 0-14-28 days of immunization was 92.28% (95% CI 85.74-95.82) [19]. After two doses, it was estimated to be very high (82.96%). Furthermore, it is important to note that during the phase 3 trial, beta, delta and gamma VOC of SARS-CoV-2 were circulating in Cuba, and a cross-protective response induced after Abdala vaccination was suggested [19]. The findings described in this study provide experimental evidence to support this notion. Consistent with this idea, we found that immunized cynomolgus monkeys elicited neutralizing antibodies against beta, delta and BA.1 omicron VOC. This was not unexpected because recent studies have shown that the S (RBD)-specific B cell response against the Wuhan strain comprises cross-neutralizing antibodies for alpha, beta, gamma, delta and omicron VOC [20,21], as well as memory cells [22]. A similar result has been reported for the Corbevax vaccine, which has an analogous composition [23]. Quantitative analysis showed that after two doses of the mRNA BNT162b2 vaccine, only a small fraction (<25%) of neutralizing antibodies derived from the natural repertoire elicited against the S protein of the Wuhan strain also cross-neutralized omicron BA.1, BA.2 variants [24]. However, further booster doses can increase the frequency of cross-neutralizing antibodies [25,26]. Several lines of evidence suggest that, although limited somatic mutations of germline B cells can result in the production of neutralizing antibodies against the original Wuhan strain [27,28], further accumulation of somatic mutations in RBD-specific IgG antibodies may play a role in the better neutralization to SARS-CoV-2 VOC over time [29,30]. In this regard, an immunization regimen of three doses may be a better choice for eliciting a broader protective response. A limitation of this study was that T cell function was not assessed. However, it is known that the highest percentage of the S (or RBD)-specific CD4+ T-cell response in convalescent or vaccinated individuals targets conserved regions of the Wuhan original strain and VOC [31]. Our findings, taken together with previous studies, suggest that vaccination with Abdala with a three-dose regimen will elicit effector and memory RBD-specific CD4+ T cells recognizing not only the Wuhan original strain, but also the VOC already tested in this study for cross-neutralization. Future experiments should address this issue directly.

Conclusion

In conclusion, the data presented here provide additional experimental evidence regarding the immunogenicity and protective capacity of the Abdala vaccine, which may explain the results observed in clinical trials and support the notion of induction of a broad cross-protective response against VOC.

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