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Design, development and preclinical evaluation of SOBERANA®02: A Cuban vaccine against COVID-19

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ABSTRACT

Introduction: Controlling the global COVID-19 pandemic depends, among other measures, on developing preventive vaccines against SARS-CoV-2. Virus infection is mediated by the interaction of the spike glycoprotein trimer, via its receptor binding domain (RBD), with the host's cellular receptor. Vaccines in use or under development seek to elicit neutralizing antibodies to block virus binding to ACE2 receptor. Antibody response to this domain is an important outcome of immunization and correlates well with viral neutralization. **Methods**: The development and preclinical evaluation of SOBERANA®02 was carried out in four fundamental stages: 1) immunogen design, 2) obtaining the RBD viral antigen by recombinant technique, 3) obtaining and characterizing RBDn-TT conjugates, and 4) evaluation of conjugates immunogenicity in laboratory animals. **Results and discussion**: Here we show that macromolecular constructs with recombinant RBD conjugated to tetanus toxoid (TT) induce a potent immune response in laboratory animals. Some advantages of immunization with RBD-TT conjugates include a predominant anti-RBD IgG immune response due to affinity maturation and long-term specific B-memory cells. Conclusions. This result demonstrated the potential of this COVID-19 conjugate vaccine candidate and enabled its advance to clinical evaluation, paving the way for other antiviral conjugate vaccines.

Keywords: SARS-CoV-2 Infection; COVID-19 vaccines; immunity; vaccines, conjugate

Diseño, desarrollo y evaluación preclínica de SOBERANA®02: Una vacuna cubana contra COVID-19

RESUMEN

Introducción: El control de la pandemia global de COVID-19 depende, entre otras medidas, del desarrollo de vacunas preventivas contra el SARS-CoV-2. La infección del virus está mediada por la interacción del trímero de la glicoproteína espiga, a través de su dominio de unión al receptor (RBD), con el receptor celular del hospedero. Las vacunas en uso o en desarrollo buscan generar anticuerpos neutralizantes para bloquear la interacción del virus con el receptor ACE2. La respuesta de anticuerpos hacia este dominio correlaciona bien con la neutralización viral. Métodos: El desarrollo y la evaluación preclínica de SOBERANA®02 se realizó en 4 etapas fundamentales: diseño del inmunógeno; obtención del antígeno viral RBD mediante técnica recombinante; obtención y caracterización de conjugados RBDn-TT y evaluación de la inmunogenicidad de los conjugados en animales de laboratorios. Resultados y discusión: Este trabajo muestra que una construcción macromolecular compuesta de RBD

recombinante conjugado a toxoide tetánico (TT) induce una respuesta inmune potente en animales de laboratorios. Algunas ventajas de la inmunización con el conjugado RBD-TT son la respuesta predominante de IgG contra el RBD debido a la maduración de la afinidad y una respuesta en memoria de células B específicas. Conclusiones, Este resultado demostró el potencial de este candidato vacunal contra COVID-19 y permitió su avance a ensayos clínicos, abriendo el camino para otras vacunas conjugadas antivirales.

Palabras clave: Infección por SARS-CoV-2; vacunas contra la COVID-19; inmunidad; vacunas conjugadas

INTRODUCTION

SARS-CoV-2 infection begins with the binding of a trimeric construction of the spike protein (S) present on the surface of the viral particles to host's cellular receptors. [1,2,3] In humans, SARS-CoV-2 uses the angiotensin-converting enzyme 2 (ACE2) cell receptor, which is expressed in respiratory epithelial cells of the airways, nasal passages, and alveoli. [2-4] The binding of protein S-composed of 1273 amino acids and highly glycosylated-to the ACE2 receptor is mediated by a fragment of the S1 subunit called the Receptor Binding Domain (RBD). [2-4] The RBD has an immunodominant character within the S protein, and in turn is the target of around 90% of the neutralizing antibodies generated in convalescents from COVID-19. [5] Internationally, two different types of protein subunit vaccines were originally designed, those based on the entire protein S [6] and those based solely on RBD as the vaccine antigen. [7,8]

However, very early on, our group and others demonstrated that the recombinant RBD obtained in cells of higher organisms is relatively poorly immunogenic, which is due in part to its low molecular weight (~30 kDa), the presence of glycans (protein-bound carbohydrates) of mammalian origin and low activation of B cells due to monovalent presentation of this monomeric antigen. This leads to a low production of RBD-specific antibodies. [8] Thus, the scientific hypothesis of this work was that the chemical conjugation of the RBD antigen to a carrier protein such as tetanus toxoid (TT) would allow obtaining a more immunogenic RBD-TT conjugate than the monomeric RBD, and therefore of greatest potential as an immunogen of an effective vaccine against COVID-19. Consequently, the objective of this research was to develop a vaccine candidate based on a conjugate of RBD to TT with the capacity to generate a strong response of neutralizing antibodies and cellular memory in laboratory animals and later in humans.

METHODS

The development and preclinical evaluation of SOBERANA®02 was carried out in four fundamental stages: immunogen design, obtaining the RBD viral antigen by recombinant technique, obtaining and characterizing RBDn-TT conjugates, and evaluation of conjugates immunogenicity in laboratory animals.

To obtain the RBD viral antigen, the recombinant expression of the RBD sequence (Arg319-Phe541-$(His)_6$) was performed in CHO-K1 host cells, supported by the CIM recombinant protein production platform: work with cells lines derived from the CHO cell line, fermentation in perfusion mode to obtain the culture broth enriched in the protein of interest, as well as the purification and characterization of glycoproteins.

Obtaining this vaccine antigen began with the cloning in a lentiviral vector of the gene coding for the RBD protein of the SARS-CoV-2 virus from aa 319 to 541, followed by codons that code for a six-histidine tag. The protein-encoding lentiviral vector RBD 319-541 was used to transduce the Chinese hamster ovary cell line CHO-K1. Subsequently, a stable line producing RBD 319-541 protein in both monomeric and dimeric forms was obtained by adaptation to chemically defined culture media and cell cloning cycles.

The amino acid sequence of the recombinant RBD was extended up to residue 541 to include Cys538, which, being unpaired, led to the antigen was obtained as a mixture of RBD monomer with Cys538 cysteinylated with another Cys from the culture medium, and dimer with two RBD units linked by an intermolecular disulfide bridge.

The RBD monomer was separated from the dimer by molecular exclusion chromatography for subsequent conjugation to TT, while the dimer was used as the immunogen of SOBERANA®01 and SOBERANA®Plus.

Production at 2 L fermenter scale resulted in RBD 319-541 protein used to obtain conjugates for preclinical evaluation. The use of this platform was strategic in the SOBERANAS Vaccines project, because it facilitated the successful transfer of the process from the development phase at a pilot scale in 2 L fermenters to the production scale in 500 L and 2000 L fermenters.

To obtain the RBDn-TT conjugates, a selective reduction procedure of Cys538, inserted in the sequence for this purpo-

se, was developed to obtain a free thiol that can be used in the conjugation to TT without affecting the other four intramolecular disulfide bridges that maintain the native three-dimensional structure of the RBD and are key to maintain its correct antigenicity (figure 1A). Various reducing agents such as ditriotreitol (DTT) and tris-(2-carboxyethyl)phosphine (TCEP) were studied, as well as protocols in which reaction time, temperature, RBD concentration and stoichiometry were varied.

Once the selective reduction of RBD was standardized and scaled up, conjugation to the TT protein (~150 kDa) was performed using the addition of thiols to maleimido groups. [9] Figure 1B shows this procedure, which was performed in several batches modifying the stoichiometry of the reaction to obtain a conjugate with 2 units of RBD per TT (RBD_2-TT) and another with 6 units of RBD per TT (RBD_6-TT). Figure 1C shows the structural representation of the RBD_2-TT and RBD_6-TT conjugates.

Once the most efficient conjugation procedure was established, the technology was scaled up under Good Manufacturing Practice (GMP) conditions. The critical operational parameters, product quality attributes including quality specifications, process and quality controls, the generation of all necessary documentation, as well as the process at a scale that currently produces about one million doses per batch were established.

In the immunogenicity evaluation stage, RBD_2-TT and RBD_6-TT conjugates, and others (not shown), were evaluated for humoral and cellular response in mice to provide preclinical evidence of which candidates were most suitable for advancement to human clinical trials.

To evaluate the functionality of the antibodies, i.e. their neutralizing character, two assays were performed with the sera of vaccinated mice: the molecular neutralization assay and the assay reporting serum dilution.

The cellular response generated for both immunogens adsorbed on alum was also studied. The specific T-cell response was also evaluated and the avidity indexes (AI) of the antibodies generated by both conjugates obtained were compared.

RESULTS AND DISCUSSION

Regarding the design of the immunogen, in July 2020, the first report of the use of monomeric RBD - obtained recombinantly in insect cells - as the immunogen of a Chinese vaccine candidate was published. [7] However, our strategy was different and focused on the design of a conjugate of RBD to a carrier protein such as TT, seeking to make it more immunogenic than RBD alone.

The elements to predict the higher immunogenicity of the conjugate were: a) its higher molecular weight favors transit from the subcutaneous tissue to the lymph nodes, where it accumulates in antigen-presenting cells and favors T-cell and antibody responses, b) its ability to more efficiently activate B cells (antibody-producing lymphocytes) due to the multivalent presentation of the viral antigen and c) the immunopotentiating effect derived from the activation of T helper cells (T hel-

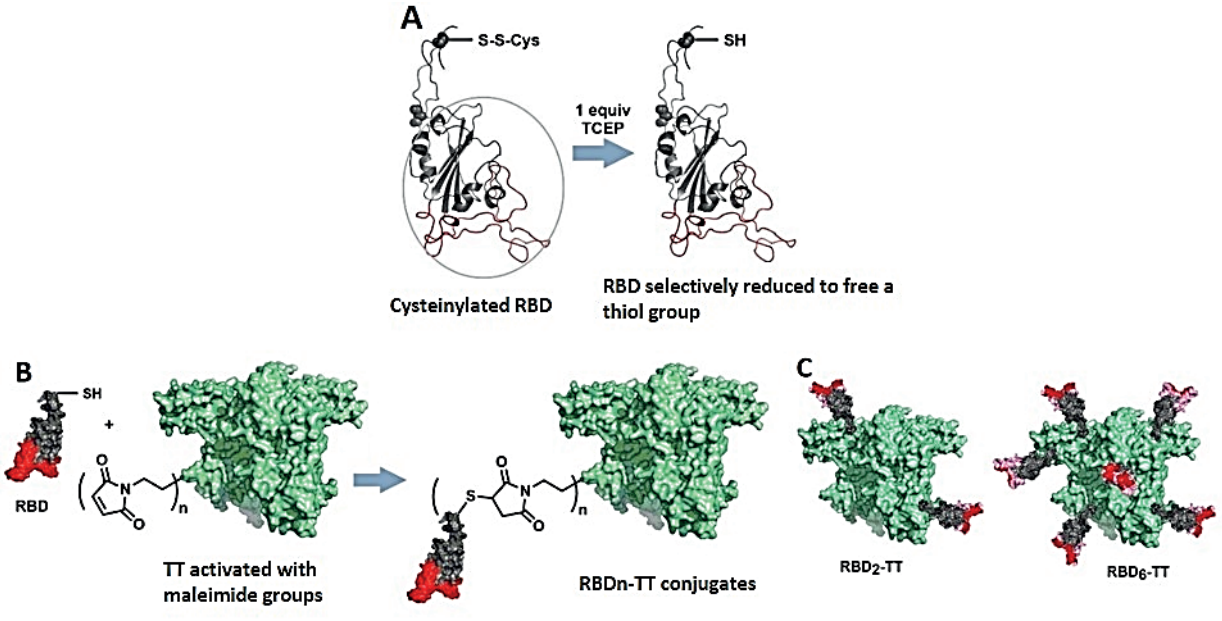

Fig. 1. A) Site-selective functionalization of RBD by intermolecular S-S bond reduction at Cys538. B) Chemical conjugation of RBD to TT. C) Structural representation of RBD2-TT and RBD6-TT conjugates.

per lymphocytes) by T epitopes present in the tetanus toxoid.

For this, the chemical conjugation of RBD to TT had to be carried out specifically through a protein site that did not affect the conformational epitopes of RBD responsible for the interaction with the ACE2 receptor, called Receptor Binding Motif (RBM), since these are essential to generate an efficient neutralizing antibody response. In addition, the viral antigen was expressed in mammalian cells to ensure that the antibody response was directed towards the polypeptide fragment of the RBM and not towards oligosaccharide chains (glycans).

The use of a TCEP equivalent under controlled reaction conditions allowed the selective reduction of Cys538 without affecting its antigenicity. Figure 2 shows the characterization of the RBDn-TT conjugates.

Figure 2A shows that the recognition by convalescent sera of the RBD reduced with TCEP is identical to the native RBD, whereas a similar reduction with DTT leads to a total loss of antigenicity, possibly by breaking other intramolecular disulfide bridges.

Figure 2B shows the preservation of the antigenicity of the conjugates, as they are recognized-even better than native RBD-by the ACE2 receptor in ELISA assays. A model RBD_6-BSA conjugate was also well recognized by convalescent sera.

The high efficiency and reproducibility of the conjugation reaction, performed with strict control of time, concentration, equivalents, etc. has been fundamental to the success of the vaccine, as this reaction has been scaled up to the gram level to produce up to one million doses in one conjugation batch.

The scale-up of the conjugation step progressed from batches of 150 thousand doses to batches of 1 million doses, which had a significant impact on the vaccine production system. Comparability was demonstrated between the different development stages of the scale-up (laboratory, pilot and industrial), which allowed an accelerated progress and, at the same time, consistency was demonstrated in the production process, which had a decisive impact on obtaining the certificate of Good Manufacturing Practices issued by CECMED and the granting of the Emergency Use Authorization for the vaccine.

The characterization studies performed on the proteins produced in each of the scales reported correct identity of primary, secondary and tertiary sequence, characteristic glycosylation of CHO cells, purity above 90% and reactivity on the ACE2 molecule, which demonstrated the quality of the processes.

Conjugates with higher RBD load were also obtained, but showed no advantage over the RBD_6-TT conjugate in preclinical evaluation.

SOBERANA 02 is the only vaccine that employs a chemical conjugation of RBD to TT to combine the advantages of multivalent presentation and the immunopotentiating effect of TT. It is worth noting that other conjugated immunogens reported as vaccine candidates have not yet been approved as a vaccine, making SOBERANA 02 the world's first conjugate vaccine against COVID-19.

From the standpoint of immunogenicity evaluating of the conjugates in laboratory animals, the RBD_2-TT and RBD_6-TT conjugates, and others (not shown), were evaluated for humoral and cellular responses in mice to provide preclinical evidence of which candidates were most suitable for advancement to human clinical trials. Figure 3A shows the immunization scheme used, in which the response of conjugates with different RBD loadings were compared with the response of monomeric RBD, all adjuvanted with alum. Figure 3B shows that RBD_6-TT/alum generated an early and high anti-RBD antibody response, which is highly desirable in times of pandemic,

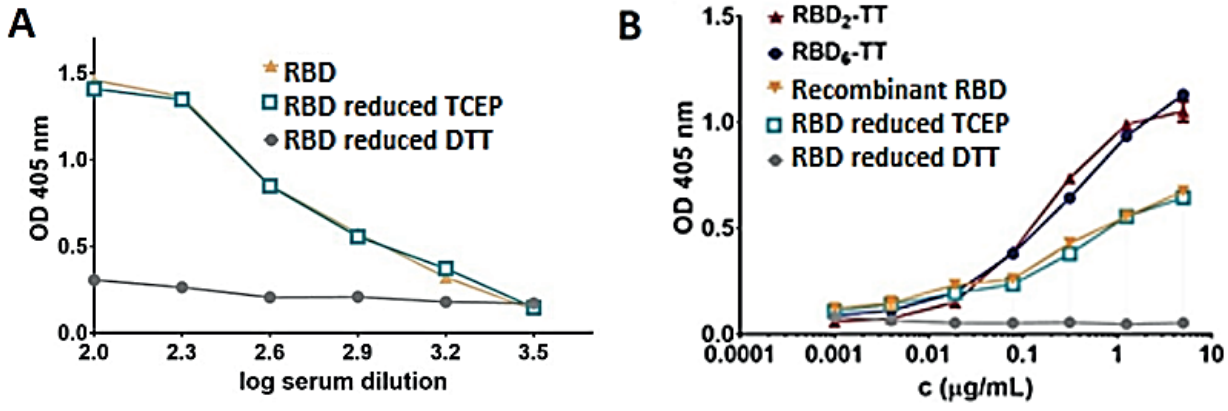

Fig. 2. Characterization of RBDn-TT conjugates. A) Recognition by convalescent sera (antigenicity) of native and reduced monomeric RBD. B) Study of the interaction of RBD-ACE2 with monomeric RBD (native and modified) and RBD-TT conjugates.

while RBD_2-TT/alum and RBD/alum required the booster dose to achieve high IgG titers. With RBD_6-TT/alum a dose study was performed with 0.5, 1 and 3 µg of the conjugate finding a dose-dependent IgG response at day 7, but at day 14 the response was very high even for the lowest dosage. The avidity indexes (AI) of the antibodies generated by both conjugates were compared, finding 81% for those of RBD_6-TT/alum and 69% for those of RBD_2-TT/alum (figure 3C). This is consistent with more pronounced affinity maturation and predicts better functionality of the antibodies produced by RBD_6-TT. In addition, a skewed Th2 immune response was observed for RBD_2-TT/alum (IgG2a/IgG1 ratio 0.54), whereas a more balanced Th1/Th2 response was found for RBD_6-TT/alum (IgG2a/IgG1 ratio 0.81) (figure 3D).

To evaluate the functionality of the antibodies, i.e. their neutralizing character, two assays were performed with sera from vaccinated mice. The first is the molecular neutralization assay, representing the serum dilution that resulted in 50% inhibition of the RBD-ACE2 interaction (mVNT50, figure 3E), and the second is the assay reports the serum dilution that resulted in 50% neutralization of the virus interaction with Vero E6 cells expressing the ACE2 receptor (cVNT50, figure 3F). Figures 3E and 3F show a higher level of neutralization at the molecular and cellular level by sera from vaccinated mice with RBD_6-TT/alum, demonstrating that the humoral response generated by this immunogen is not only higher but also of better quality.

Finally, the cellular response generated for both immunogens adsorbed on alum was studied. A splenocyte transfer experiment from vaccinated to naive mice was performed. The response of specific T cells was also evaluated. In addition, the avidity indexes (AI) of antibodies generated by both conjugates were compared and a biased Th2 immune response was observed for RBD_2-TT/alum.

Conclusions

It was demonstrated that the neutralizing and cellular antibody response in laboratory animals is significantly higher with the use of RBD-TT conjugated immunogens than with monomeric RBD, thus proving the working hypothesis. From the studied conjugates, RBD_6-TT showed an earlier, higher and more neutralizing humoral response than RBD_2-TT, making it the immunogen of choice for advancement to human clinical trials.

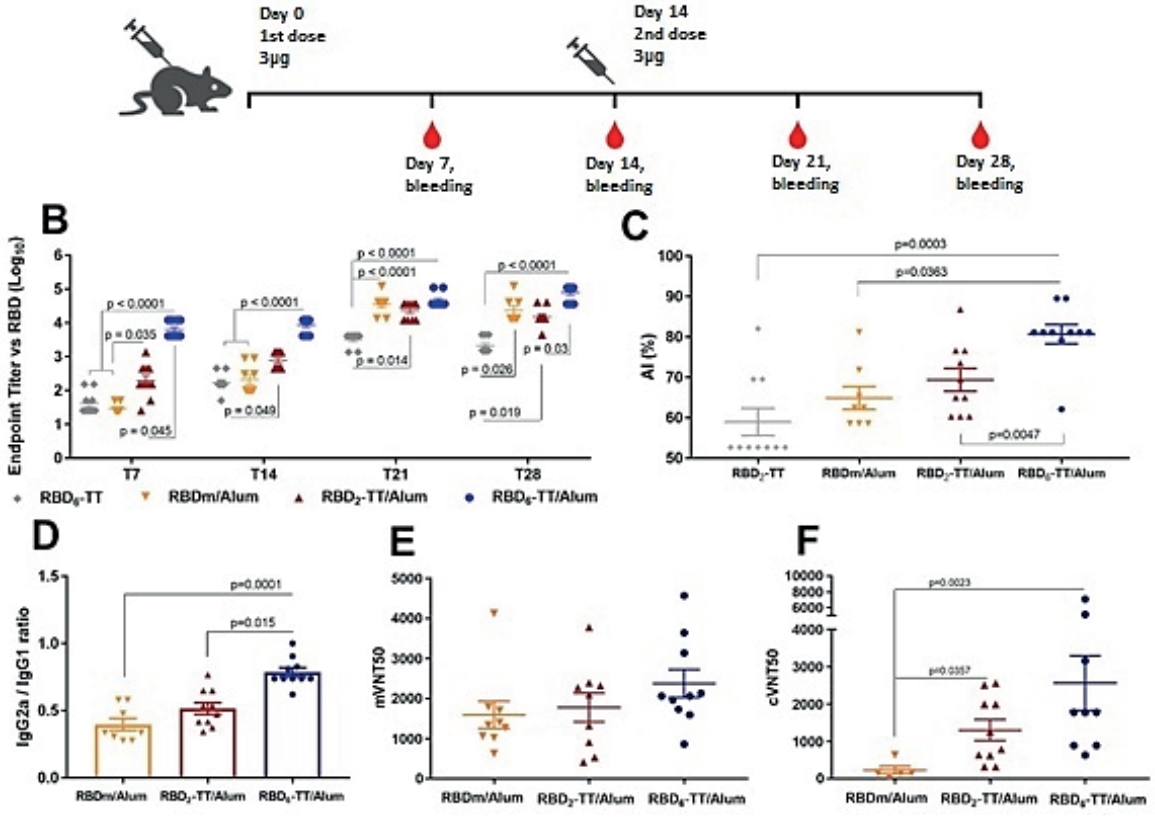

Fig. 3. A) Immunization scheme of BALB/c mice. B) Specific anti-RBD antibodies generated at days 7, 14, 21, and 28. C) Avidity index of antibodies generated at day 28. D) IgG2a/IgG1 antibody ratio. E) Inhibition assay of the RBD-ACE2 molecular interaction. F) Neutralization assay of virus interaction with Vero E6 cells containing the ACE2 receptor.

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Competing interests

The authors declare no financial conflicts of interest. Yuri Valdés Balbín, Darielys Santana Mederos, Sonsire Fernández Castillo, Manuel García Ricardo, Laura Marta Rodríguez Noda, Belinda Sánchez Ramírez, Ubel Jesús Ramírez González, Reynaldo Oliva Hernández, Daniel García Rivera, Tammy Boggiano Ayo, Eduardo Ojito Magaz, Daniel García Rivera, Vicente Guillermo Verez Bencomo are co-inventors on provisional SARS-CoV-2 vaccine patents (Cu 2020-69).

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- Investigation: Darielys Santana Mederos, Lauren María Quintero Moreno, Sonsire Fernández Castillo, Laura Marta Rodríguez Noda, Rocmira Pérez Nicado, Claudia Ofelia Acosta Grogues, Yanira Méndez Gómez, Manuel García Ricardo, Tays Hernández García, Gretchen Bergado Báez, Franciscary Pi Estopiñán, Anet Valdés Zayas, Tania Carmenate Portilla, Ubel Jesús Ramírez González, Reynaldo Oliva Hernández, Jean Pierre Soubal Mora, Raine Garrido Arteaga, Félix Cardoso San Jorge, Mario Landys Chovel Cuervo, Humberto González Rodríguez, Mildrey Fariñas Medina, Tamara Hernández Salazar, Juliet M. Enríquez Puertas, Enrique Noa Romero, Anamary Suárez Batista, Cheng Fang, Luis Ariel Espinosa Rodríguez, Yassel Ramos Gómez, Luis Javier González López, Yanet Climent Ruiz, Gertrudis Rojas Dorantes, Ernesto Relova Hernández, Yanelys Cabrera Infante, Sum Lai Losada, Kalet León Monzón, Françoise Paquet, Guang-Wu Chen, Lila Rosa Castellanos Serra
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