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SARS-COV-2 Prevention Strategy Receptor Binding Domain Loaded with Chitosan Nanoparticles Enhance Antibody Production

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Abstract

Background: Corona virus disease 2019(COVID-19), caused by the severe acute respiratory syndrome virus (SARS-CoV-2), heavily relies on the angiotensin-converting enzyme 2 (ACE2) receptor for cell entry. The receptor-binding domain (RBD) of the virus plays a critical role in this process and is considered a key target for neutralizing activity. The RBD can be produced efficiently and effectively to induce a neutralizing immune response. Chitosan (CS), a widely used vaccine adjuvant, shows promise as an adjuvant/carrier in vaccine delivery.

Objective: To investigate the potential of CS nanoparticles (CS-NPs) as an alternative adjuvant to stimulate specific anti-RBD IgG antibody production efficiently and with reduced toxicity compared to alum.

Methods: Antigens were prepared by loading RBD protein onto CS-NPs, followed by measuring the released RBD protein using high-performance liquid chromatography. Rabbits were vaccinated, and the resulting antibody response was detected using competitive enzyme linked immunosorbent assay.

Results: The use of CS-NPs as an adjuvant loaded with the RBD antigen demonstrated the ability to induce a substantial titer of anti-RBD IgG antibodies when administered to rabbits over a course of three doses with two-week intervals. Comparatively, using alum as an adjuvant resulted in higher antibody titers with the same vaccination schedule.

Conclusion: It was observed that CS-NPs as an adjuvant could efficiently stimulate specific anti-receptor-binding domain IgG antibodies while exhibiting less toxicity compared to alum

Keywords: SARS-CoV-2, vaccine adjuvant, chitosan nanoparticles, RBD, specific IgG antibody.

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List of abbreviations: ACE2 = Angiotensin-converting enzyme 2, BSA = Bovine serum albumin, COVID-19 = Corona virus disease 2019, CS = Chitosan, CS-NPs = Chitosan nanoparticles, ORF = Open reading frames, RBD = Receptor-binding domain, SARS-CoV-2 = Severe acute respiratory syndrome virus

Introduction

The name "coronavirus" comes from the fact that coronaviruses are enclosed positive sense RNA viruses with spike-like projections on their surface that give them a crown-like appearance under an electron microscope. Their diameters range from 60 nm to 140 nm [(1)].

Forten open reading frames (ORFs) make up the severe acute respiratory syndrome virus (SARS-CoV-2) genome, and 16 nonstructural proteins (nsp 1–16) are encoded by two-thirds of them [(2)]. The remaining one-third encodes four structural proteins—spike (S), envelope (E), membrane (M), and nucleocapsid (N), of

which spike promotes SARS-CoV entrance into host cells — as well as nine accessory proteins (ORF). Angiotensin converting enzyme 2 (ACE2), a cellular receptor, is directly contacted by Spike's receptor-binding domain (RBD) [3]. ACE2, a required receptor for SARS-CoV-2, allows the virus to enter cells [4]. It has a wide tissue tropism and is extremely contagious, which is probably what keeps the pandemic going [5].

In order to engage host cells and begin infection, the RBD of the spike protein on the viral surface is a crucial epitope and is therefore a good candidate target antigen for the production of neutralizing antibodies. Ninety percent of serum neutralizing activity in people targets RBD because it is simple to make, effective, and expresses up to 100 times more than spike trimer [6].

Virus-induced immunological reactions trigger antibody-mediated defenses. When the B cells mature into plasma cells, which then manufacture antibodies tailored to a viral antigen, the T cells provide assistance. An antibody with a neutralizing nature is effective in completely preventing the virus from entering host cells to avoid infection, and it also plays a very strong protective role in the later stages of infection and prevents infection relapse [7].

The construction of nanostructured scaffolds for stimulating powerful immune responses known as vaccines has seen substantial advancements [8]. RBD-based subunit vaccines have been limited by the RBD peptide component's weak inherent immunogenicity, despite the fact that it is simple to create and extremely stable. This restriction can be removed by persistent co-administration with a strong and effective adjuvant [9].

The use of adjuvants as a tactic to boost the immune response to particular vaccinations, notably those for influenza, has been the subject of numerous research. Since chitosan (CS) has well-known biosafety and mucosal adsorption-promoting qualities, it has been employed as an adjuvant for vaccine administration [10].

A partially deacetylated form of chitin is chitosan, because of its distinctive qualities, including biodegradability, biocompatibility, and biological activity. It claimed that under typical physiological conditions in vitro; chitosan demonstrated a significant binding affinity to ACE2 and SARS-CoV-2S-RBD [11].

Aluminum salts are frequently employed as additives in vaccines to enhance and activate the immune system. These aluminum-based adjuvants have a long-standing history of use, but there have been concerns regarding potential adverse effects, toxicity, and the possibility of excessive vaccination [12].

This study aimed to find a novel approach of vaccine development to SARS-CoV-2 by using encapsulated bacteriophage loaded with RBD through testing the immunizing potential pre-clinically in lab animals

Methods

Study design

The purpose of this study was to use chitosan loaded with the SARS-CoV-2 spike protein to experimentally immunize subjects. The laboratory work was conducted at the Central Public Health Laboratory under the Ministry of Health, while the in vivo phase took place at the Veterinary Drugs Research and Production Center under the Ministry of Industry and Minerals. The antigen preparation protocol involved utilizing CS nanoparticles (CS-NPs) loaded with the RBD protein. The released RBD protein was measured using high-performance liquid chromatography (HPLC). Following that, rabbits were vaccinated, and the presence of specific anti-RBD IgG antibodies was detected using a competitive ELISA assay.

Loading of CS-NPs with NPs with bovine serum albumin (BSA) and RBD

A Tris-HCl buffer solution with a concentration of 0.02 M was prepared by dissolving it in 1 ml of distilled water. The standard protein, BSA, and the RBD were mixed separately with the

0.02 M Tris-HCl buffer solution, and a pH of 8.5 was chosen for this mixture. CS-NPs were mixed with the 0.02 M Tris-HCl buffer solution, and a pH of 5.5 was chosen for this mixture. Equal volumes of 500 µl of BSA and 500 µl of RBD, which were previously mixed separately with 500 µl of CS-NPs, were combined. The resulting mixture was then incubated overnight at 37°C [(11)]. The binding affinity of the BSA, RBD, and CS-NPs mixture was determined using the Bradford method [(13)].

The in vitro release study of BSA and RBD

The assay employed in this study aimed to quantify the amount of protein released from CS-NPs when bound with BSA or RBD. The experimental protocol used was based on the method described by Rahimzadeh et al. [(14)] with some modifications. Firstly, known concentrations of BSA (0.25, 0.5, 1, 2, 4, 8 µg/ml) were measured using HPLC to construct a BSA standard curve. Then, 1 ml of CS-NPs was loaded with 8 µg of BSA, and a 10 ml suspension was prepared in a glass tube. The mixture was thoroughly mixed and incubated at 37°C with shaking overnight. Samples (500 µl) were collected at predetermined time points (2, 4, 6, 8, 10 days) to obtain five aliquots of the suspension. Each aliquot was centrifuged, and the supernatant was transferred to separate clean tubes. The concentration of BSA released from the complex over time was determined by HPLC analysis. The same experiment was performed using RBD, where different concentrations of RBD in various solutions were tested. The optimal solution was selected based on the concentration of RBD antigen released from the complex over time.

To measure the concentrations of free RBD protein released over time from CS-NPs loaded with RBD, HPLC analysis was performed using a SYKAM model with a C18 column (250mm x 4.6 mm, 5 µm particle size) at room temperature. UV detection at 272 nm was utilized during the analysis.

Binding of RBD protein with adjuvant aluminum hydroxide

For comparison purposes, aluminum hydroxide, which serves as a standard adjuvant, was used in contrast to the newly designed adjuvant known as CS-NPs. In the experimental procedure, the purified recombinant RBD protein was combined with aluminum hydroxide at a ratio of 1:20. The mixture was then incubated overnight at 35°C, following a modification of the method described in reference [(15)].

Immunization program of Rabbit

Rabbit of type (Albino), males, aged 1-1.5 years old with 2-2.5 kg weight were divided into 3 groups with total number of 9 rabbits according to table (1).

Table 1. Study groups of rabbits according to type of adjuvant

Groups No.	Type of immunogen	Concentration of RBD per single dose (µg)	Volume of single dose (µl)	Number of doses	Route of admission
1	RBD+CS-NPs	30	600	3	I.M
2	RBD+Alum	30	600	3	I.M
3	CS-NPs	-	600	3	I.M

Immunoassay of anti RBD

Blood samples were obtained by Cardiac puncture 5-10 ml of blood were obtained and the concentration of anti RBD IgG was determined in serum using a competitive enzyme-linked immunosorbent assay (ELISA) kit manufactured by Elabscience/USA following manufacturers instructions.

Results

The concentrations (µg/ml) of released BSA from suspension of CS-NPs loaded with BSA (8 µg/ml) were measured by HPLC within 10 days, (Table 2).

While the results of released RBD concentrations with time for each 4 samples are shown in table (3).

The concentrations of RBD antibody were measured in rabbit serum by ELISA according to their groups classified in table (1) depending on type of adjuvant and there were significant differences (P <0.0001) comparing to control groups as shown in figure (1).

Table 2. The Concentrations (µg/ml) of released free BSA during 10 days

Duration time	Day 2	Day 4	Day 6	Day 8	Day 10
Concentration (8 µg/ml)	0.31	0.4	0.52	0.37	0.26

Table 3. The concentrations of released free RBD with time

Suspension tube number	RBD conc. (µg/ml)	RBD pH value	Phage-CS-NPs pH value	Concentrations of free RBD (µg/ml) After 3 days incubation A	After 7 days incubation B
1	8	6	10	0.08	0.2
2	2	6	10	0.5	1.1
3	8	10	5.5	1.6	3
4	2	10	5.5	1.0	1.6

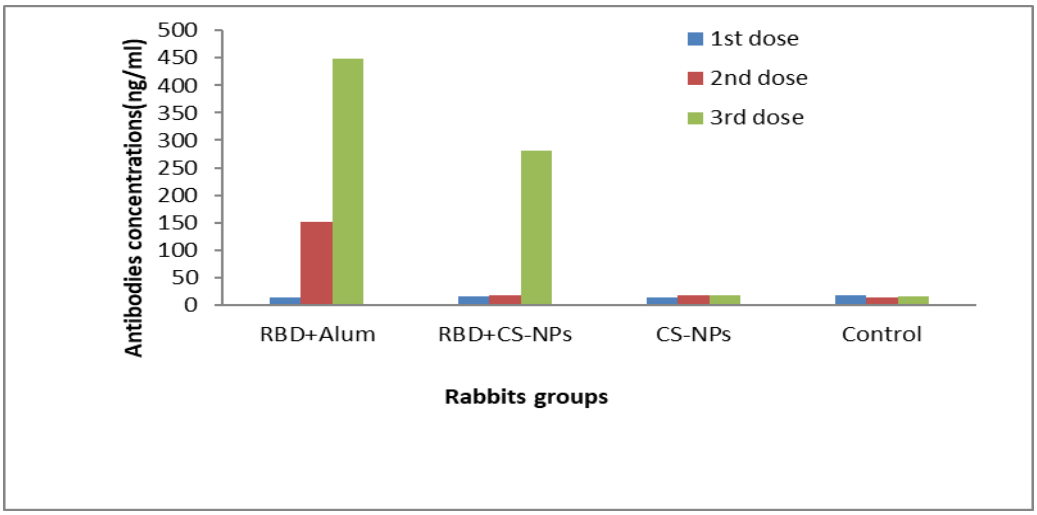

Figure 1. Receptor-binding domain Antibody concentrations of rabbits group

Discussion

There is an urgent requirement for an effective preventive vaccine against SARS-CoV-2, the virus responsible for the COVID-19 pandemic. One crucial aspect of the infection process involves the interaction between the (RBD) of the spike protein of SARS-CoV-2 and the host cell receptor (ACE2) [16].

Various studies have demonstrated that a recombinant vaccine containing the RBD of the spike protein can elicit a strong and functional antibody response in mice, rabbits, and non-human primates [17].

The estimated concentrations of released BSA and RBD in this study were measured by HPLC, the in vitro study shows that BSA released from CS-NPs complex in relatively slow constant pattern and the same occurred with RBD in 4 different solutions, this finding showed that the CS-NP could acts as good adjuvant for the in vivo immunization trial in lab animal, the liberation of miner concentrations of RBD may trigger the immune response to produce specific immunoglobulin for that antigen. The recombinant RBD protein is proved as a potent antigen and a novel adjuvant is in demand for the effective stimulation of adaptive immunity. Therefore, to improve the efficacy of the vaccine and seek a novel adjuvant that can stimulate both humoral and cellular immunity, The recombinant RBD vaccine for SARS-CoV-2 was chosen to be administered intramuscularly and mucosally using chitosan a cationic nanocarriers as powerful adjuvants [18].

Regarding the in vivo immunization, the selection of rabbits as a source of target-specific antibodies was made since they have clear benefits over mice. They can elicit a significant immune response against antigens or epitopes that are poorly immunogenic or tolerated in mice because they make higher affinity antibodies than mice can [19].

In the initial two groups of rabbits, the administration of 30 µg of RBD adjuvanted with either CS-NPs or alum resulted in the production of anti-RBD IgG antibodies. The rabbits received three doses of the vaccine, with a two-week interval between each dose, administered intramuscularly. The last group of rabbits served as a control and was injected with CS-NPs alone. The study demonstrated that the RBD vaccine formulated with CS-NPs as an adjuvant induced a robust antibody response ($P < 0.0001$). However, the antibody titer generated with CS-NPs was lower compared to that achieved with alum, which is attributed to the use of alum as a stronger adjuvant. Notably, CS-NPs exhibited lower toxicity compared to alum, as CS-NPs are derived from a natural compound. In a previous study, rabbits were immunized with a 20 µg dose of RBD combined with either liposomes or alum, administered intramuscularly on day 0 and day 21. The post-immunization sera demonstrated the presence of anti-RBD IgG antibodies on day 21, with liposomes exhibiting a boosting effect that resulted in approximately 10-fold higher antibody levels on day 42 compared to alum [20].

In conclusion, in this study, novel vaccine delivery strategies were developed, utilizing CS-NPs as an adjuvant. The use of CS-NPs as an adjuvant proved to be both safe and effective in generating protective immune responses, particularly in terms of producing specific antibodies against the RBD protein when administered to rabbits. Although the specific anti-RBD IgG antibody titer was lower when using CS as the adjuvant compared to conventional adjuvants like alum, CS demonstrated lower toxicity.

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Author contribution

Mahdi: conducted the experimental work and writing. Dr. Abdulamir and Dr. Taha supervised the work.

Conflict of interest

There are no conflicts of interest.

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