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Development of COVID-19 Recombinant Subunit Vaccine and Adjuvant

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Abstract: *At present, vaccination is the only effective and low-cost way to prevent and treat COVID-19. At present, the vaccines vaccinated on a large scale are inactivated COVID-19 vaccines, which have the risk of insufficient immunogenicity, low neutralizing antibody production level and short maintenance time, and incomplete pathogen inactivation leading to adverse reactions. The recombinant subunit vaccine can effectively overcome the problem of poor safety of inactivated virus vaccines by using immune epitope proteins or peptides as recognition epitopes, and with the assistance of appropriate adjuvants, it can produce high levels of neutralizing antibodies and maintain good immune memory. This paper will review the application of recombinant protein antigen of COVID-19 virus and various adjuvants, and prospect the future application of recombinant protein vaccine.*

Keywords: *COVID-19, Subunit vaccine, Adjuvant*

1. Introduction

Through the efforts of scientific researchers and pharmaceutical companies around the world, different kinds of vaccines have been put into use. Among them, recombinant subunit vaccine has become a hot research topic because of its good biosafety. In order to enhance the immunogenicity of recombinant subunit vaccine, the research and development of new adjuvants have emerged. In this paper, the recombinant proteins and adjuvants of COVID-19 virus under development are systematically reviewed.

2. Infection mechanism and immune target S protein of COVID-19 virus

COVID-19 differs from other coronaviruses in that there are surface glycoproteins (spike glycoproteins) on the surface of the virus, which are highly glycosylated in the process of synthesis and assembled into trimers on the virion surface to form its special surface Spike structure. COVID-19 genome consists of 14 open reading frames, two-thirds code 16 kinds of nonstructural protein (nsp1-16), be made replication transcription complex. The remaining third encodes 9 accessory proteins (ORFs) and 4 structural proteins, among which, in addition to Spike proteins, small envelope proteins, matrix proteins and nucleocapsid proteins[1], as shown in Figure 1.

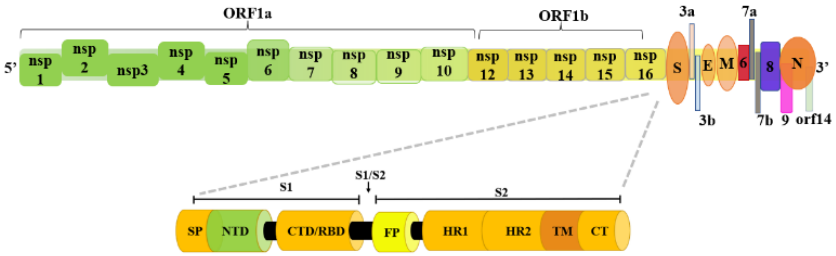

Figure 1: Structural composition of COVID-19 genome and S protein

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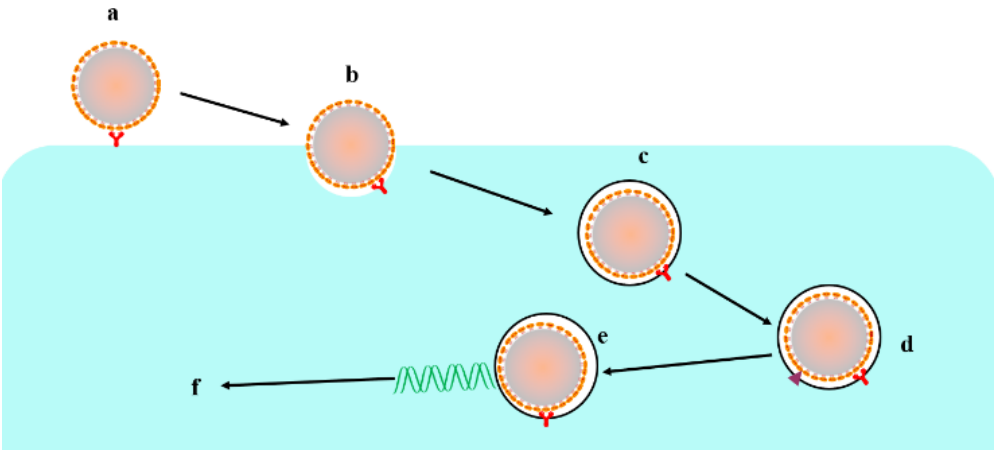
Spike proteins are divided into S1 and S2 subunits. The S1 subunit contains a signal peptide and two domains, N-terminal domain (NTD) and C-terminal domain (CTD), and the receptor binding domain (RBD) is in CTD. The S2 subunit contains essential elements for membrane fusion, including an inner membrane fusion peptide (FP), two hepta-peptide repeats (HR), a transmembrane domain (TM) and a cytosolic domain(CT)[2]. Viral particles first bind to the ACE2 receptor on the host cell membrane through S proteins on their surface, and are subsequently ingested into endocytosomes. S proteins on the surface of viral particles are cleaved into S1 and S2 subunits by CathepsinL and TMPRSS2 provided by the host lysosome after ingestion into endocytic vesicles. TMPRSS2 promotes viral entry to the plasma membrane surface.Cathepsin L, on the other hand, can activate S protein spikes in endosomes, which can compensate for entry into cells lacking TMPRSS2[3]. When the viral genome is released into the host cytoplasm, ORF1a and ORF1b are translated into viral replicase proteins, which are subsequently cleaved into individual replicase components (NSP) to form viral RNA-dependent RNA polymerases. NSPS rearrange the ER into double-membrane vesicles to facilitate replication of viral genomes and subgenomic RNAs, which are translated into helper proteins and viral structural proteins to facilitate the formation of viral particles, as shown in Figure 2.



(a) The S protein protruded from the surface of the virus binds to ACE2, a receptor on the cell membrane. (b) The virus is endocytosed by the cell. (c) The virus endocytosis into the endocytosome. (d) S protein is cleaved and processed into S1 and S2 subunits by proteases in lysosomes. (e) Membranes fuse and viral RNA enters the cell. (f) Virus replication

Figure 2: The process of host infection by COVID-19 virus

Previous studies have shown that S proteins in coronaviruses not only determine their tissue tropism and host tropism, but also serve as the target antigen of neutralizing antibody produced by the immune system of the infected host[4]. Receptor binding region on S protein is directly involved in host receptor recognition during virus infection, and its amino acid variation will significantly affect virus virulence and infectivity, which is the main reason why Spike protein can become a key target for the development of vaccines and therapeutic antibodies.

3. Overview of adjuvants used in recombinant protein vaccines

3.1. Aluminium adjuvant

The history of the application of aluminum adjuvant and coronavirus recombinant protein can be traced back to the outbreak of SARS virus. When aluminum adjuvant, SARS S protein or RBD are used as vaccines, they will significantly enhance humoral immune response. The immune effect can be confirmed by the high titer of neutralizing antibody produced by mice, the level of serum antibody IgG1and long-term memory B cells. A study by Pasteur Institute in Shanghai showed that after the RBD domain protein, S1 subunit, spike protein trimer of COVID-19 and aluminum adjuvant were used to immunize mice, all three successfully generated neutralizing antibodies in mice. However, at the same antigen dose, RBD and spike trimer elicited more durable and higher titer neutralizing antibodies in mice than S1 vaccine[5].

However, it is worth mentioning that the immune response of inactivated COVID-19 or S protein subunit vaccines mainly tends to Th2 type, which is characterized by increased eosinophil and

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inflammatory infiltration, and aluminum adjuvant will aggravate this response. When Al (OH)3 is used as adjuvant of vaccine, it lacks Th1 guided CD4+T cell response and CD8+T cell response, which is its deficiency. In addition, when aluminum adjuvant binds to protein antigens, precipitation will occasionally form, which will also shield the binding sites on the antigen surface, which will affect the immune effect of the vaccine to a certain extent, which is a key problem to be solved.

3.2. Emulsion adjuvant

Compared with the aluminum adjuvant that lacks the ability to mediate cellular immunity, the emulsion adjuvant can trigger a more balanced immune response by improving antigen uptake, recruiting immune cells, and promoting the activation and migration of antigen presenting cells. An experiment at the University of Hawaii in the United States evaluated the immune effect of Covaccine^{HT} adjuvant compounded with S1 subunit of spike protein of novel coronavirus[6]. The experimental results showed that after immunizing mice for a single time (day 14), serum antibody analysis showed that the specific IgG antibody titer of Covac group was significantly higher than that of aluminum adjuvant group (the titer level of aluminum adjuvant group was close to the baseline), and the results of microsphere immunoassay data also confirmed that immunization with S1 subunit of COVID-19 and Covaccine^{HT} adjuvant could induce a high level of antigen-specific IgG response. As an adjuvant of TLR4 agonist, Covaccine^{HT} mainly causes Th1 type immune response. Through the analysis of IgG subclasses, it was found that covac group showed a variety of immunoglobulin reactions composed of IgG1, IgG2a and IgG2b subclasses. The levels of these subclasses of immunoglobulins further increased after the second vaccination. The aluminum adjuvant group and the antigen group mainly produced Th2 immune responses. In addition, only Covac group can induce neutralizing antibodies against novel coronavirus. The results of plaque reduction neutralization experiment show that the titers of $PRNT_{90}$ and $PRNT_{50}$ in this group are (1:1620), and the neutralization titers of $PRNT_{90}$ and $PRNT_{50}$ in other groups are less than 20. Therefore, Covaccine^{HT} as an adjuvant, plays an obvious role in enhancing immunogenicity of the antigen.

In the study completed in January 2021, Katharina et al. also compared the immune effects of full-length S protein as antigen with CAF®01 (DDA/TDB), aluminum adjuvant, and AddaVax™ as adjuvant, in which CAF®01 is a cationic liposome and AddaVax™ is an oil-in-water squalene nanoemulsion[7]. Specific lgG antibody responses were clearly observed 7 days after immunization in the AddaVax™ group, which was much higher than that in the antigen alone group. Pseudovirus neutralization tests also showed that virtually no neutralizing antibody titers were detected except in the AddaVax™ group.There was little difference in specific lgG antibody titers among all adjuvant groups after the cessation of the experiment at 45 days of immunization. Neutralizing antibody titers were observed in both adjuvant groups, with an ID50 titer of 86 to 4063 in the adjuvant group and a slightly higher neutralizing antibody titer in CAF®01 than in AddaVax™, both of which were higher than those in the aluminum adjuvant group.In this study, the immunological efficacy of three adjuvants was evaluated using the trimeric S protein as an antigen, with the AddaVax™ group producing germinalcenters (GC) the fastest, as confirmed by staining of GCB cells and follicular T helper cells (Tfh) 7 days after immunization.Finally, all adjuvants affected GC initiation times in response to S protein to varying degrees, with the AddaVax™ group responding fastest, but antibody responses, including the ability to neutralize novel coronavirus, were basically similar after 45 days of immunization.

3.3. Toll-like receptor

The combination of Toll like receptor agonist and aluminum salt adjuvant can exert the dual adjuvant effect. In a recently published paper, etsuronanishi and other researchers systematically evaluated the immune effect of RBD protein mixed with various adjuvants and aluminum adjuvants as a vaccine in adult and old mice[8]. After two immunizations, the serum antibody analysis results of adult mice showed that compared with pure antigen, PRR agonist significantly enhanced the anti RBD antibody titer and the ability to inhibit RBD binding to human ACE2. Al + CpG showed the highest induction of total IgG, IgG1 and IgG2a, and the IgG2a: IgG1 ratio was much higher than that of other adjuvants. In the long-term immunogenicity evaluation after 210 days, the Al+ CpG group also maintained high inhibition of hACE2 RBD binding, and the level of inhibition binding was much higher than that of the other groups.The results of serum analysis of aged mice after immunization were similar to those of adult mice, and also Al+CpG group showed the highest titers of hACE2-RBD inhibition and neutralizing antibody levels.It is worth noting that the aged mice immunized with Al+CpG and AS01B adjuvant did not show weight loss within 4 days, while the aged mice immunized with RBD without adjuvant or other adjuvant

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groups rapidly lost about 10% of body weight within 4 days. The results of lung tissue virus titer detection and pathological analysis also showed that Al+CpG and AS01B adjuvant could effectively protect aged mice from novel coronavirus infection.In this study, CpG2395, which is class C, was used in combination with aluminum adjuvant to better balance the reaction between Th1 and Th2. The adsorption of CpG by aluminum adjuvant also extended its half-life to a certain extent, and improved the disadvantage of easy degradation of oligontide, so that it could play the role of immune adjuvant in the local area for a long time.In addition, Al+CpG also induced strong GC response in adult mice and aged mice. Germinal center response was the basis for inducing high-quality and durable B cell response, and follicular helper T cells (Tfh) were the key regulator of GC response.GCB cells, IgG1+GCB cells, Tfh cells and proliferative Ki67+Tfh cells were observed in both mice immunized with Al+CpG twice.AS01B adjuvant is A liposome adjuvant composed of monophosphoryl lipid A (MPL) and saponin QS-21. At the same time, the combination of AS01B adjuvant and CpG was also compared. AS01B has been approved for use in the recombinant herpes zoster vaccine Shingrix (CHO cells).It is specifically used for the prevention of herpes zoster in adults aged 50 years and over. When aged mice were immunized, the HACE2-RBD binding inhibition ability (57±2%) and neutralizing antibody titer (2344±7) of Al+CpG group were higher than those of AS01B (14±3%;117±4), which is surprising given that AS01B has shown excellent immune effects in the elderly[9].

Toll like receptor 7/8 agonists are also a kind of gradually emerging vaccine adjuvants. TLR7, TLR8 and TLR9 are located on endosomes of many cell types. 3M-052, an adjuvant developed by 3M, is a synthetic TLR-7/8 agonist with 18-c fatty acyl chain small molecules, belonging to imidazoloquinolines.3M-052 is generally adsorbed on aluminum adjuvants, which is similar to CpG-ODN[10]. MariaPino et al. conducted a study on 3M-052 adsorbing on alum as an adjuvant in 2021[11], also using RBD receptor-binding domain as an immunogen. Compared with aluminum adjuvant alone, the 3M-052 and aluminum adjuvant group promoted higher levels of anti-RBD binding effector antibodies and COVID-19 neutralizing antibodies, elevated Th1-biased $CD4^+$T cell responses and increased $CD8^+$T cell responses.In the subsequent live virus challenge experiment, 3M-052 and aluminum adjuvant also showed strong immune effect. On the second day after live virus challenge, total RNA in the lavage fluid of mice was detected. 3M-052/aluminum adjuvant group could protect animals from COVID-19 infection. The alveolar tissue of four out of five mice was virus-free on day 4 and completely virus-free on day 5, and similar results were found in lung pathology.Compared with the aluminum adjuvant + antigen group and the antigen alone group, the 3M-052+ aluminum adjuvant group had less pulmonary changes in RMS.Nanda et al. also studied the application of 3M-052 in combination with aluminum adjuvant, but the target antigen of this study was the trimer of RBD[12]. The study also compared two adjuvants, aluminum adjuvant and Al-3M-052.The antibody response was measured at the third and sixth week after immunization. The RBD-binding antibody titer of 1.7×10^4 was generated in the Al-3M-052 group, which was close to the level of the aluminum adjuvant group, but there was no significant difference between the two groups.However, after twice immunization with Al-3M-052 adjuvant combined with RBD trimer, a high level of binding antibody response was induced. After enhanced immunization, the neutralization titers of Al-3M-052 group and aluminum adjuvant group were increased nearly 100 times compared with those after the first immunization, and the titer of Al-3M-052 group reached 1.7×10^6. Aluminum adjuvant group reached 1.9×10^4. The antibody induced by immunization also showed strong viral neutralizing activity. The geometric mean value of neutralization titer was 7000 in Al-3M-052 group and 66 in aluminum adjuvant group, indicating that RBD binding titer and neutralizing antibody titer were directly related.Moreover, at 2 weeks after booster immunization, the ratio of IgG2a/IgG1 of RBD-binding antibody in Al-3M-052 group was significantly higher than that in aluminum adjuvant group, and the former showed a Th1-biased immune response compared with the latter. Immunoevaluation in macaque monkeys also demonstrated that Al-3M-052 induced higher levels of neutralizing antibody titers and Th1-type $CD4^+$T cell activation than aluminum adjuvant group.

3.4. Nano adjuvant

Nanoparticles can carry antigens in different ways. For example, antigens are embedded in NPs to deliver and release antigens.The antigen can also be displayed on the surface of particles by adsorption or chemical coupling to realize pathogen imitation and other functions. For example, cross-presentation of antigen can be realized by controlling parameters such as particle size and surface charge of NPs.NPs can also convert soluble antigens into granular antigens, which is conducive to the recognition and uptake by immune cells such as macrophages and dendritic cells, and promote the activation and maturation of antigen presenting cells (APCs) and the activation of T/B cells, greatly enhancing the immune effect of vaccines[13].Therefore, nano-immune adjuvants have become a research hotspot in recent years, and nano-adjuvants have also been widely studied in the development of COVID-19 vaccines.

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A novel approach to COVID-19 vaccine development using nanoparticles is the encapsulation or self-assembly of nanoparticles using S proteins or viral subunits.Walls et al. designed a subunit nanoparticle vaccine based on RBD protein to display RBD multivalently on the outer surface of two-component protein nanoparticle I53-50.I53-50 is a designed, 28nm wide, 120-subunit complex with icosahedral symmetry, consisting of trimer (I53-50A) and pentamer (I53-50B)[14]. This nanoparticle is mainly constructed by the in vitro assembly of I53-50A and I53-50B.The advantage is that multivalent antigen presentation is possible, with each nanoparticle displaying 60 copies of RBD. The nanoparticle was characterized with a particle size of 30nm (22%), monodisperse, well-structured, and high stability.Subsequent immune evaluation also showed that RBD-I53-50 nanoparticles stimulated strong neutralizing antibody responses in BALB/ C and human immune lineage mouse models. The elicited neutralizing antibody titers could induce high levels of antibody titers at a dose lower than 5 times the S protein antigen.The antibody titer level was 1~2 orders of magnitude higher than that induced by S protein. After two immunizations, the results of live virus challenge were consistent with the trend of antibody titer.Importantly, similar titers of neutralizing antibodies were induced at doses of 0.9μg and 5μg, indicating that displaying RBD on I53-50 nanoparticles could save the amount of antigen and lay a good foundation for vaccine industrialization.

PolymerNPs are also a widely used delivery system today. Due to their homogeneous and stable chemical structure, synthetic polymers have unique advantages, such as plasticity, biocompatibility and degradability.Polymer nanoparticles can be constructed in a controllable manner in terms of size, surface charge and structure, so as to realize the controlled release and protection of drugs.PLGA is an FDA-approved biodegradable synthetic polymer for human drug delivery.It has been shown that when antigen is embedded in PLGA nanoparticles, it can remain undegraded under physiological conditions for more than 4 weeks.In addition, they can promote antigen internalization by APCs .In addition, PLGA-based nanoparticles have been reported to improve the immunogenicity of several conventional and recombinant vaccines for humans and animals.

Therefore, polymer nanoparticles are a potential vaccine adjuvant delivery system, which can reduce the amount of antigen, be safe and reliable, and have broad prospects in combating novel coronavirus.

4. Conclusion

The S protein contains multiple B cell and T cell epitopes, which can induce neutralizing antibodies and long-term immunity, indicating that this protein can be used as a major candidate for vaccine antigen. Mutation, however, can avoid inducing neutralizing antibodies mediated by vaccination protection, according to reports S protein antibody dependent enhancement happen as the immunogen phenomenon, this may be associated with COVID-19 mutation. Because subunit vaccine is a part of the pathogen protein as antigen, so the immunogenicity is weak. In addition, the different expression system will also affect the immunogenicity of the vaccine. The expressed antigen protein may be different from the natural conformation of the pathogen protein, which will also affect the immune effect of the vaccine. Although ADE effect has not been reported for different fragment antigens based on S full-length proteins except for the full-length Spike protein antigen, the present results only show no ADE effect in mouse and nonhuman primate models, which does not exclude the risk of ADE effect in human immunity. At present, the research on subunit vaccine is very hot, and the potential ADE risk still needs careful attention.

Based on this, it is necessary to carefully design and test newly developed vaccines to evaluate which virus mutations can escape antibody mediated neutralization and which mutations can significantly affect the immune efficacy of currently used vaccines. At the same time, new adjuvants were developed to further enhance the immunogenicity of COVID-19 antigens.

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