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Purified coronavirus spike protein nanoparticles induce coronavirus neutralizing antibodies in mice

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ABSTRACT

Development of vaccination strategies for emerging pathogens are particularly challenging because of the sudden nature of their emergence and the long process needed for traditional vaccine development. Therefore, there is a need for development of a rapid method of vaccine development that can respond to emerging pathogens in a short time frame.

The emergence of severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003 and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) in late 2012 demonstrate the importance of coronaviruses as emerging pathogens. The spike glycoproteins of coronaviruses reside on the surface of the virion and are responsible for virus entry. The spike glycoprotein is the major immunodominant antigen of coronaviruses and has proven to be an excellent target for vaccine designs that seek to block coronavirus entry and promote antibody targeting of infected cells.

Vaccination strategies for coronaviruses have involved live attenuated virus, recombinant viruses, non-replicative virus-like particles expressing coronavirus proteins or DNA plasmids expressing coronavirus genes. None of these strategies has progressed to an approved human coronavirus vaccine in the ten years since SARS-CoV emerged. Here we describe a novel method for generating MERS-CoV and SARS-CoV full-length spike nanoparticles, which in combination with adjuvants are able to produce high titer antibodies in mice.

1. Introduction

Coronaviruses infect a range of mammals and birds, causing respiratory tract and gastrointestinal tract infections. Coronaviruses were known to cause severe and, therefore, economically important diseases in chickens [1] and pigs [2], but, while a number of coronaviruses were known to infect humans, the symptoms are usually mild in healthy adults, akin to a common cold, and only rarely cause more severe pneumonia. In 2003, however, severe acute respiratory syndrome coronavirus (SARS-CoV) emerged, causing 8273 confirmed infections, of which 775 resulted in death [3–5]. Most of the cases were linked to China, Hong Kong and Singapore, with the only major outbreak outside of this area occurring in Toronto, Canada. SARS-CoV had a zoonotic origin, having emerged from bats, via civet cats, to infect humans [6,7]. Although there have been no reported cases of SARS-CoV infection in humans after this, a recent study has shown that the parental virus still exists in bats in China [8].

In late 2012, a novel betacoronavirus named Middle East Respiratory Syndrome Coronavirus (MERS-CoV) was identified in a sample from a severe respiratory infection patient in The Kingdom of Saudi Arabia (KSA) [9,10]. Since then, 238 cases have been positively identified, of which 92 have resulted in death (www.who.org). All of the cases have been linked to countries on or near the Arabian peninsula (KSA, Jordan, Qatar, Egypt, Oman and United Arab Emirates). Cases in other parts of the world, notably Europe, involved recent travelers to the Middle East region or were closely linked with people who did [11]. Patients infected with MERS-CoV present at the hospital with symptoms consistent with a severe lower respiratory tract infection and, in some cases, develop kidney failure. MERS-CoV is closely related to bat coronaviruses found in China, Europe and Africa, suggesting a zoonotic origin, similar to SARS-CoV, however the reservoir of MERS-CoV has not yet been identified.

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Coronaviruses are enveloped viruses with large single-stranded positive sense RNA genomes which encode 4 major structural proteins: spike (S), membrane (M), envelope (E) and nucleocapsid (N) [12]. The S protein is a type I trans-membrane glycoprotein expressed on the surface of coronaviruses that is responsible for receptor binding and virion entry to cells [13]. The location of S on the virion surface, the role of S in binding to coronavirus receptors and the finding that S can induce neutralizing antibodies *in vivo* [14] have made it an attractive target for vaccine development strategies [15,16].

Previous efforts to create a vaccine for SARS-CoV have utilized a number of approaches, but none is currently licensed for use and a recent study of four putative SARS-CoV vaccines yielded negative results [17]. Initial studies suggested that whole inactivated SARS-CoV could be used as an effective vaccination [18–20], however further work has suggested that the level of protection induced by inactivated SARS-CoV is incomplete and fails to prevent SARS-CoV symptoms, while also inducing increased eosinophilia in vaccinated animals [17,21]. Therefore, the most likely candidates for coronavirus vaccine platforms are based on spike subunits [22,23], recombinant viruses expressing SARS-CoV proteins [24–26], DNA plasmids expressing SARS-CoV proteins [27–29] or virus-like particle (VLP) based vaccines [30–34], however all of these approaches come with their own safety concerns and approval processes. There are currently no approved vaccines for MERS-CoV, but early studies using a modified vaccinia virus and replication deficient MERS-CoV have been shown to induce antibodies capable of neutralizing MERS-CoV *in vitro* [35,36]. Ideally, vaccines for highly pathogenic viruses, including coronaviruses, should be able to be made rapidly, on demand and in conjunction with approved adjuvants using approved techniques [37].

The emergence of both SARS-CoV and MERS-CoV highlight the importance of coronaviruses as potential human pathogens that can emerge at any time. Therefore, rapid methods for treatment and vaccination are required for this important group of viruses. In this study we describe a novel method for creating SARS-CoV S and MERS-CoV S nanoparticles that in conjunction with adjuvant are able to induce a neutralizing antibody response in mice.

2. Materials and methods

2.1. Cell and viruses

Vero E6 cells were originally obtained from the American Type Culture Collection (ATCC) and maintained in Minimal Eagles Medium (MEM; Corning) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; SAFC), 1% L-Glutamine (Gibco) and 1% Penicillin/Streptomycin (Gemini Bio-Products). *Spodoptera frugiperda* Sf9 insect cells (ATCC CRL-1711) were maintained as suspension cultures in HyQ-SFX insect serum free medium (HyClone, Logan, UT) at $27 \pm 2\,^{\circ}C$.

Mouse adapted SARS-CoV (MA15) has been previously described [38] and was grown in Vero E6 cells and stored at $-80\,^{\circ}C$. MERS-CoV (Jordan) was obtained from the NIH in conjunction with AFHSC-GEIS and NAMRU-3, with special assistance from Dr. Mohareb. All experiments with live virus were performed under biosafety level 3 conditions at the University of Maryland, Baltimore.

MERS-CoV (Jordan) Spike protein is 99.8% identical to the MERS-CoV (Al Hassa1) Spike protein sequence used in the nanoparticle cloning. We consider MERS-CoV (Al Hassa1) and MERS-CoV (Jordan) to be homologous in their Spike proteins. SARS-CoV (Urbani) Spike protein is 99.9% identical to the SARS-CoV (MA15) Spike protein sequence used in the nanoparticle cloning. We consider SARS-CoV (Urbani) and SARS-CoV (MA15) Spike proteins to be homologous.

2.2. Recombinant baculovirus

The MERS-CoV S protein sequence was from isolate Al-Hasa_1_2013 with NCBI accession #AGN70962. The SARS-CoV S protein sequence was from Urbani strain with NCBI accession #AAP13441. The genes were codon optimized for optimal expression in insect cells and biochemically synthesized for MERS-CoV S (Genscript, Piscataway, NJ) and SARS-CoV S (Geneart AG, Regensburg, Germany). Full length S genes was cloned between BamHI – HindIII sites in pFastBac1 baculovirus transfer vector plasmid (Invitrogen, Carlsbad, CA) under the transcriptional control of the *Autographa californica* Multiple Nuclear Polyhedrosis Virus (AcMNPV) polyhedrin promoter. Recombinant baculovirus construct was plaque purified and master seed stocks prepared, characterized for identity, and used to prepare working virus stocks. The titers of baculovirus master and working stocks were determined by using rapid titration kit (BacPak Baculovirus Rapid Titer Kit; Clontech, Mountain View, CA). Recombinant baculovirus stocks were prepared by infecting Sf9 cells at a low multiplicity of infection (MOI) of $\leq$0.01 plaque forming units (pfu) per cell and harvested at 68–72 h post infection (hpi).

2.3. Recombinant MERS and SARS S protein

MERS-CoV and SARS-CoV S protein antigens were produced in Sf9 cells at $2–3 \times 10^6$ cells/ml infected with specific recombinant baculovirus. Infected Sf9 cells were incubated with continuous agitation at $27 \pm 2\,^{\circ}C$ and harvested at 68–72 hpi by centrifugation at $4000 \times g$ for 15 min. S proteins were extracted from cellular membranes with a non-ionic detergent and insoluble material removed by centrifugation at $10{,}000 \times g$ for 30 min. S proteins oligomers were purified using a combination of anion exchange, affinity and size exclusion chromatography. During purification the majority of the detergent is removed allowing S trimers to form higher ordered protein–protein micellular nanoparticles. Purified S nanoparticles were 0.2 micron filtered and stored $-80\,^{\circ}C$.

2.4. Analytical methods

MERS-CoV and SARS-CoV S were analyzed by SDS-PAGE using 4–12% gradient polyacrylamide gels (Invitrogen), stained with GelCode Blue stain reagent (Pierce, Rockford, IL) and quantified by scanning densitometry using OneDscan system (BD Biosciences, Rockville, MD). Purified S proteins were tested for total protein concentration (BCA bicinchoninic acid protein assay, Pierce Biochemicals) and particle size by dynamic light scattering using ZETASizer Nano (Malvern Instruments, PA) using standard, manufacturer recommended methods.

2.5. Electron microscopy

Purified MERS-CoV and SARS-CoV S proteins were adsorbed by flotation for 2 min onto a freshly discharged 400-mesh carbon parlodion-coated copper grid (Poly-Sciences, Warrington, PA). The grids were rinsed with buffer containing 20 mM Tris, pH 7.4, and 120 mM KCl and negatively stained with 1% phosphotungstic acid, then dried by aspiration. Virus-like particles were visualized on a Hitachi H-7600 transmission electron microscope (Hitachi High Technologies America, Schaumburg, IL) operating at 80 kV and digitally captured with a CCD camera at 1 K × 1 K resolution (Advanced Microscopy Techniques Corp., Danvers, MA).