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REVIEW

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Clinical development of SpikoGen®, an Advax-CpG55.2 adjuvanted recombinant spike protein vaccine

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ABSTRACT

Recombinant protein vaccines represent a well-established, reliable and safe approach for pandemic vaccination. SpikoGen® is a recombinant spike protein trimer manufactured in insect cells and formulated with Advax-CpG55.2 adjuvant. In murine, hamster, ferret and non-human primate studies, SpikoGen® consistently provided protection against a range of SARS-CoV-2 variants. A pivotal Phase 3 placebo-controlled efficacy trial involving 16,876 participants confirmed the ability of SpikoGen® to prevent infection and severe disease caused by the virulent Delta strain. SpikoGen® subsequently received a marketing authorization from the Iranian FDA in early October 2021 for prevention of COVID-19 in adults. Following a successful pediatric study, its approval was extended to children 5 years and older. Eight million doses of SpikoGen® have been delivered, and a next-generation booster version is currently in development. This highlights the benefits of adjuvanted protein-based approaches which should not overlook when vaccine platforms are being selected for future pandemics.

KEYPOINTS

- SpikoGen is a more traditional COVID-19 vaccine comprising SARS-CoV-2 spike protein extracellular domain formulated with Advax-CpG adjuvant
- SpikoGen differs from the Novavax vaccine in major ways including its use of the soluble secreted spike protein ECD rather than nanoparticle formulation and the use of a different adjuvant
- SpikoGen demonstrates robust protection against homologous and heterologous SARS-CoV-2 strains in hamster, ferret and non-human primate challenge models
- SpikoGen induces broadly cross-neutralizing antibodies, but still protects even after these antibody levels wane
- In a pivotal Phase 3 clinical trial, SpikoGen reduced the risk of severe infection by 77.5% and was not associated with myocarditis, thrombosis or any other adverse safety signals
- SpikoGen received an Emergency Use Authorization in the Middle East on 6 October 2021, making it the first recombinant spike protein vaccine to achieve this milestone
- Eight million doses of SpikoGen vaccine have been safely delivered to date
- Protein-based vaccines have a long history of reliability and safety

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Introduction

COVID-19 is caused by lung infection with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2).[1] Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes a constellation of clinical outcomes from asymptomatic infection to respiratory failure and death.[2] The evolution of immune-escape SARS-CoV-2 variants continues to represent a global challenge[3] even though the pandemic phase has passed. While novel COVID-19 vaccines were rolled out with unprecedented speed, many challenges remain. New immune-escape variants continue to reinfect vaccinated as well as previously infected individuals. There remains a need for vaccines able to prevent SARS-CoV-2 infection and transmission, a weakness of most if not all current vaccines.[4]

Now the pandemic phase has passed, it is a good time for reflection on what might have been done better with respect to the development and roll out of the COVID-19 vaccines.[5] This review specifically focuses on the experience with SpikoGen® vaccine as an example of one of the first successful protein-based COVID-19 vaccines. In particular, it seeks to answer the question of whether protein-based vaccines have been rendered obsolete by newer mRNA approaches. Certainly, during the early phase of the pandemic, protein-based vaccines were overlooked in favor of the newer mRNA and adenoviral vector approaches, but the reasons for this were financial and political rather than science based. Currently, large investments are being made in many countries for construction of mRNA manufacturing facilities but is this money well spent? How much of the mRNA euphoria is driven by reality and how much by

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hype?[6] Did protein-based vaccine approaches get a fair hearing in countries like the United States?

In addition to speed of development, an ideal pandemic vaccine technology should be low cost, scalable, temperature stable, well tolerated and safe, and provide robust and durable protection against both serious infection and transmission. Inactivated whole-virus COVID-19 vaccines were widely used in developing countries, with the main sources being China (Sinopharm) and India (Bharat Biotech).[7] These were shown to be safe and well tolerated, although their efficacy against infection proved to be modest, they did not prevent transmission, lacked durability and, required BSL3 manufacturing capability, which is extremely limited in most parts of the world. Other major COVID-19 vaccine approaches were mRNA or adenoviral vector based. However, this is not intended as a critical review of the mRNA or adenovirus vector platforms, which dominated Western markets during the early stages of the pandemic.[8] Unfortunately, adenovirus vector vaccines were associated with high reactogenicity and some lethal thrombotic events and have now been taken off the market.[9,10] Similarly, mRNA vaccines were associated with high reactogenicity and inflammatory myocarditis,[11] provided relatively short-lived protection, required frequent boosters,[12] were expensive and were not stable except at very low temperatures. This indicates that these newer technologies are not ideal pandemic solutions.

Adjuvanted subunit vaccines presented an alternative well-established pandemic platform, but their potential was largely overlooked in Western countries during the pandemic. However, recombinant protein-based COVID-19 vaccines have now been licensed for human use in most countries. SpikoGen® vaccine was the first recombinant spike protein trimer-based vaccine to achieve regulatory licensure with emergency use approval by the Iranian FDA in early October 2021. This was just 18 months or so from the declaration of the pandemic by the World Health Organization (WHO). Other recombinant spike protein vaccines subsequently licensed include Novavax's Nuvaxoid® vaccine[13] and Sanofi's VidPrevtyn Beta vaccine.[14] Interestingly, all three of these recombinant vaccines are manufactured in insect cells using baculovirus transient infection, highlighting the strength of this platform for rapid and scalable recombinant protein production. A number of other protein-based receptor-binding domain (RBD) vaccines were licensed in Cuba, India and other countries.[15] These RBD-based vaccines induce antibodies that block RBD-ACE2 binding whereas the larger spike protein trimer vaccines, such as SpikoGen®, induce a much broader spectrum of antibodies against other important regions of the spike protein including the N-terminal domain (NTD), fusion peptide and stalk domain, all of which can help suppress virus infectivity.[16] This broader antibody repertoire induced by full-length spike protein versus the RBD could be of major importance for cross-protection against new variants as most immune-escape viral mutations are localized in the RBD region. The larger spike protein vaccines may also induce a broader diversity of T cell epitopes. During the pandemic, emergency use authorizations were expedited of a range of new vaccine technologies. This resulted in a massive global roll-out of novel mRNA and adenovirus vector vaccine technologies. Where does this leave the future of more traditional adjuvanted recombinant protein vaccine approaches? mRNA vaccines may be faster to produce than traditional protein-based vaccines,[17] but SpikoGen® generated its initial Phase 1 trial data in September 2020 just weeks behind the lead mRNA and adenoviral vector vaccines.[18] SpikoGen's development was hampered by a lack of resources and funding, rather than any slowness of thetechnology itself. After completing its pivotal Phase 3 clinical trial, SpikoGen® received its first market authorization in Iran in early October 2021, just 10 months after the first mRNA and adenoviral vector vaccine approvals. This 10-month delay in time to market was driven by lack of resources and supply chain failures, and hence is not proof that protein expression being inherently slower than mRNA or adenoviral vector technologies. Arguably, given equivalent resources, recombinant protein vaccines could have been developed in a very similar time frame to the mRNA and adenoviral approaches.

Each vaccine technology presents its own challenges. Protein-based vaccines had additional challenges not faced by viral vector or mRNA vaccines, including the need to develop techniques for protein purification and to perform structural characterization of the expressed protein.[19] It is not possible to measure such parameters for mRNA or viral vectors which induce protein expression *in vivo*. Another challenge for recombinant protein-based approaches is low immunogenicity.[20] However, this can be overcome by addition of a suitable adjuvant. In recent years, multiple new adjuvants have been introduced alongside existing aluminum salt and oil emulsion adjuvants.[21] New adjuvants include ISS1018, a toll-like receptor (TLR)-9 agonist in Heplisav® hepatitis B vaccine,[22] Matrix M, a saponin-based adjuvant in Nuvaxoid COVID-19 vaccine,[23] the alum-CpG adjuvant in Corbevax® RBD vaccine,[24] Alhydroxyquim adjuvant in Bharat biotech's inactivated COVID-19 vaccine approved in India,[25] ASO1B, an adjuvant containing a liposomal mixture of QS21 and monophosphoryl lipid A (MPL) in Shingrix® shingles vaccine[26] and Mosquirix® malaria vaccine,[27] AS04, an adjuvant comprising MPL absorbed to aluminum phosphate in Fendrix® hepatitis B vaccine,[28] and Vaxine's own Advax-CpG55.2 adjuvant in SpikoGen® vaccine.

Advax-CpG55.2 is a proprietary adjuvant formulation combining Advax, particles of plant-derived delta-inulin polysaccharide, with CpG55.2 oligonucleotide, a potent human TLR-9 agonist. CpG55.2 is the first licensed human medicinal product designed by artificial intelligence.[29,30] Advax-CpG adjuvant has been shown to enhance vaccine immunogenicity and protection across a broad range of animal models[31–34] including in vaccines against severe acute respiratory syndrome (SARS) coronavirus and the Middle East respiratory syndrome (MERS) coronavirus.[35] Advax-CpG adjuvanted SARS vaccine not only provided robust protection but also prevented vaccine-enhanced disease seen with other SARS vaccines.[36]

SpikoGen® vaccine design

The key antigen component in SpikoGen® vaccine is a spike protein extracellular domain (ECD) antigen derived from the original Wuhan strain sequence. Despite the absence of the

transmembrane domain it still self-assembles into a native trimer structure.[37] The SARS-CoV-2 genome sequence became available in NCBI (accession number: NC 045512) in mid-January 2020, allowing the structure of SARS-CoV to be used as a template to model the SARS-CoV-2 spike protein. We identified SARS-CoV-2 as having significant homology to SARS-CoV. We then performed structural homology modeling using the SARS-CoV structure (PDB-ID 6ACC) to obtain a 3D structure of SARS-CoV-2 spike protein. To help identify the putative cellular receptor for SARS-CoV-2, the crystal structure of human ACE2 was retrieved, and docked against human ACE2 protein. This predicted human ACE2 as the entry receptor for SARS-CoV-2 spike protein and confirmed the spike protein as an ideal target for vaccine design based on our experience with developing an effective vaccine against SARS CoV. Molecular dynamic simulations were performed on various modified spike protein ECD vaccine constructs to help select a suitable vaccine candidate while also being used to predict species susceptibility to SARS-CoV-2 infection.[38]

SpikoGen® formulation and analytical testing

An insect cell codon-optimized expression cassette encoding the spike protein ECD with a His tag and stabilizing mutations was designed and cloned into pFASTBac1 to generate baculovirus following standard procedures.[32] Recombinant baculovirus was expanded in Sf9 insect cells to P3 and then used to infect Trichoplusia ni cells for protein expression. The recombinant spike protein ECD was purified by nickel affinity chromatography. Prior to the COVID-19 pandemic, Advax-CpG adjuvant had already been shown to be safe and effective, including in clinical trials of influenza [Clinicaltrials.gov; NCT03945825; NCT03038776] and hepatitis B [Clinicaltrials.gov; NCT01951677] vaccines. This made Advax-CpG a strong candidate for inclusion in our recombinant protein-based COVID-19 vaccine. Importantly, Advax-CpG adjuvant previously showed beneficial effects in vaccines against SARS and MERS coronaviruses.[35,36] This animal immunogenicity data supported the decision to proceed with development of the recombinant spike protein ECD vaccine formulated with Advax-CpG55.2 adjuvant.[35,36]

In addition to a suitable adjuvant, many other challenges had to be solved when developing a recombinant protein-based vaccine. This included maximizing upstream protein yields while developing a downstream purification process that yielded a pure, stable and consistent protein product. Alongside optimization of the protein expression system, a large suite of analytical methods had to be rapidly developed for in-process testing and final product characterization and release.[39] Many methods for structure characterization, purification and quantification including ELISAs, and Western blots rely on availability of monoclonal antibodies. Such tools and reagents were not available at the start of the pandemic. Hence, the team needed to source and in some cases internally generate polyclonal and monoclonal antibodies needed for assay development. Another key reagent that needed to be internally generated was recombinant human ACE2 protein for use in developing a spike protein binding ELISA assay and a surrogate virus neutralization assay.[40] Spike protein pseudotyped lentivirus virus had to be produced for each of the emerging variants of concern for use in pseudotype virus neutralization assays for vaccine immunogenicity assessment as part of *in vivo* potency assays. Finally, a wide range of protein characterization tests including SDS gel electrophoresis, Western blots, size-exclusion chromatography, glycan composition analysis, glycan linkage-type analysis, peptide sequencing by mass spectroscopy, HDX-MS, negative stain EM, monoclonal antibody mapping, ACE2 binding, and accelerated stability studies, were needed to better understand the structure and behavior of the recombinant spike protein making up SpikoGen® vaccine.[39]

SpikoGen® pre-clinical studies

The safety and immunogenicity of SpikoGen® vaccine was first assessed in BL6 and BALB/c mice. Immunization with Advax-CpG55.2-adjuvanted recombinant spike protein ECD induced spike-specific IgG and IgM antibodies with potent virus neutralization activity. Neutralization activity was measured by either surrogate virus neutralization assays based on inhibition of RBD binding to human ACE2, lentivirus pseudotype neutralization assays, or wildtype virus neutralization assays.[32] These assays confirmed that the recombinant spike ECD produced in T.ni insect cells when combined with Advax-CpG55.2 adjuvant was a good vaccine immunogen. The insect cell expression system enabled eukaryotic-like glycosylation of the spike protein thereby ensuring its structural integrity, whilst mitigating the risk of adventitious virus contamination associated with mammalian cell systems. It also provided good yields of protein at 10–40 mg/L thereby ensuring a competitive cost per dose and was able to be rapidly scaled.[41] Immunization with spike protein alone or mixed with alum adjuvant produced a Th2-biased immune response whereas spike protein formulated with Advax-CpG adjuvant induced a balanced T cell response characterized by both Th1 and Th2 cytokines.[42] This was considered important as both SARS CoV and SARS-CoV-2 viruses inhibit Th1 interferon pathways as a mechanism of immune escape.[43] Countering these viral effects by priming a strong memory Th1 and interferon response should be beneficial, with CD8+ T cells playing a critical role in silencing virus-infected cells.[44] Advax-CpG adjuvant induced a mixed Th1/Th2 response to SARS-CoV-2 recombinant spike protein in mice resulting in increased IgG1, IgG2 and IgG3 production and an increased frequency of IL-2, IFN-γ and TNF-α secreting T cells.[32] Sera from SpikoGen®-immunized mice cross-neutralized the B.1.1.7 (Alpha or UK) variant. The CpG55.2 component in SpikoGen® also induced cytotoxic T cells able to kill spike peptide-labeled target cells, *in vivo*.[32]

SpikoGen® vaccine was next assessed for its ability to protect against SARS-CoV-2 infection in relevant animal models. The ferret challenge model was initially used as there was a high degree of uncertainty what might be the best animal model for the new SARS-CoV-2 virus. In the ferret model, two doses of SpikoGen® vaccine given intramuscularly several weeks apart provided complete lung protection and also completely stopped day 3 nasal virus shedding, an exciting finding as it suggested a possible impact on virus transmission.[32]

Ferrets turned out to not be a good model of severe infection as they did not develop major clinical symptoms or weight loss when infected with SARS-CoV-2. SpikoGen® vaccine was next tested in the Golden Syrian hamster model which had advantages over ferrets as the SARS-CoV-2 virus replicates efficiently in naïve hamster lungs and the animals develop signs of clinical disease including weight loss and lung pathology.[45] To assess protection, hamsters were immunized intramuscularly with SpikoGen® vaccine, either once or twice 3 weeks apart. Sera were obtained 2 weeks after the final vaccination for measurement of neutralizing antibodies, and the hamsters were then challenged intranasally with a homologous wild-type SARS-CoV-2 virus isolate, USAWA1/2020. SpikoGen®-immunized hamsters achieved high serum neutralizing titers against the homologous SARS-CoV-2 virus. Naive control hamsters lost an average of 6.4–6.8% body weight during the first two days post-challenge, whereas SpikoGen®-immunized animals exhibited significantly reduced weight loss. There was a strong negative correlation ($R = -0.8$) between the serum neutralization titer, pre-challenge, and cumulative weight loss, post challenge. Both the dual- and single-dose vaccine regimens protected against lung infection and pathology, with a high correlation between serum neutralizing antibody levels and lung protection. Other studies have similarly shown a strong correlation between serum neutralizing antibody levels and protection against systemic disease.[46,47] The viral load in lungs and nasal turbinate strongly correlated with weight-loss and lung pathology scores and serum neutralizing antibody levels correlated with reduced viral load in lung and nasal turbinates.[45] Interestingly, throat swab viral loads did not predict disease severity and showed a poor correlation with weight loss or lung scores. There was also a poor correlation between serum neutralizing antibody levels and throat viral loads. This suggests that serum antibody do not mediate mucosal protection but instead largely act to prevent the virus entering the lower respiratory tract.

SpikoGen® vaccine was also tested for safety and efficacy in the macaque non-human primate (NHP) model. As seen in hamster and ferret models, two doses of Advax-CpG adjuvanted spike protein vaccine induced robust serum neutralizing antibody levels against the homologous virus in the NHPs and protected against lung virus replication and lung pathology.[48] An interesting feature of this study was that the virus challenges were performed approximately 3 months after the second vaccine dose at a time when serum neutralizing antibody levels had fallen to undetectable levels in most of the immunized animals. Nevertheless, all the immunized animals, bar one, were completely protected against any lung virus replication. The one exception was an elderly female who was clinically well but showed a low level of lung virus day 3 post-challenge.

Preclinical studies in hamsters and NHP were also undertaken to assess the capacity of SpikoGen® vaccine, which was based on the original Wuhan spike protein, to protect against heterologous virus variants. The hamster study showed that two doses of SpikoGen® vaccine could protect hamsters against both the beta or delta variants, with the best protection been seen with a trivalent version of SpikoGen® that contained Wuhan, beta and gamma spike proteins.[49] This study also confirmed for the first time that SpikoGen® vaccine could prevent transmission from immunized animals to naïve co-housed sentinels. A more recent NHP study also showed that a bivalent SpikoGen® version containing Wuhan and Mu spike proteins, after three intramuscular doses, protected against the Omicron BA.5 variant, despite the animals having no detectable serum neutralizing activity against BA.5 pre-challenge.[50] The same study also showed that the original monovalent SpikoGen®vaccine when given as a course of two intramuscular doses followed by two oral doses protected against Omicron BA.5 infection. These animal data highlight that the original monovalent SpikoGen® vaccine, despite containing Wuhan spike protein, continues to provide cross-protection against newer Omicron strains such as BA.5. Given this protection was seen in the absence of BA.5 neutralizing antibodies, this suggests either that SpikoGen® vaccine is inducing protective non-neutralizing antibodies, or protecting by another mechanism such as induction of protective T cell responses.[51]

A consistent finding across the animal studies was that SpikoGen® vaccine reduced nasal virus shedding and in hamster studies this was confirmed to translate to prevention of virus transmission from an infected host to a naïve co-housed sentinel.[49] SpikoGen® vaccine was extremely well tolerated in all tested species, with no major adverse events. Given its strong safety and efficacy profile in multiple species, SpikoGen® technology was selected by the Australian Zoos Association to immunize susceptible zoo species including lions, tigers, and non-human primates against COVID-19.[52] A bivalent version called SpikeVet™ that contained Wuhan and Mu spike protein CD with Advax-CpG55.2 adjuvant was administered as a course of two or three immunizations, with 867 vaccine doses being administered to 354 zoo animals, drawn from 38 species in total, thereby potentially representing perhaps the largest OneHealth pandemic vaccine studies ever attempted. SpikeVet™ was found to have an excellent safety profile in the zoo species tested with the low rate of minor adverse effects and the absence of major adverse events. The types of local and systemic minor adverse effects observed in zoo animals were broadly comparable to those reported for other protein-based vaccines in humans.[53]

SpikoGen® human clinical trials

An Australian first-in-man Phase 1 clinical trial of Covax-19®, the earliest version of SpikoGen®, was conducted in 40 adult participants commencing in mid-2020, mere months into the pandemic (clinicaltrials.gov; NCT04453852). This confirmed the safety of the adjuvanted recombinant spike protein vaccine platform. Mainly mild local and systemic adverse events were short-lived and commensurate with the side effects of existing licensed vaccines.[54] Next, a randomized, placebo-controlled, double-blind Phase 2 trial was conducted in the Middle East on 400 adult participants randomized 3:1 to receive two doses of 25 μg SpikoGen® 3 weeks apart or saline placebo (clinicaltrials.gov; NCT04944368). SpikoGen® vaccine induced humoral and T cell responses against spike protein in the majority of participants and was not associated with any adverse safety signals.[18] The most common solicited adverse events were injection site pain, fatigue and headache, and these were

predominantly graded as mild and transient. Two weeks post the second vaccine dose, 87% of immunized participant sera neutralized SARS-CoV-2 virus at titers > 1:32. Baseline seronegative subjects achieved neutralizing antibody levels post 2-doses that were 3.3-fold higher than levels in convalescent infected individuals. The SpikoGen® group, 2 weeks post the second dose, exhibited increased antigen-stimulated T cell proliferation and interferon gamma production. Baseline seropositive participants showed a large amnestic SARS-CoV-2 antibody response after just a single SpikoGen® dose, consistent with having B cell memory due to previous infection. Based on the positive Phase 2 safety and immunogenicity results, SpikoGen® vaccine was advanced into a pivotal Phase 3 trial conducted as a placebo-controlled, double-blind trial in participants aged 18 to 50 years.[18] The primary endpoint was efficacy in preventing symptomatic PCR-confirmed SARS-CoV-2 infection, with a total of 16,876 participants undergoing randomization on 3:1 basis to receive two intramuscular doses 3 weeks apart of SpikoGen® (25 mcg) or saline placebo. A single interim analysis was pre-specified upon accrual of a minimum of 74 primary endpoints, representing 50% of the 147 endpoints planned for a final analysis. The vaccine safety data in the Phase 3 trial mimicked that of the Phase 1 and 2 trials and was characterized by transient mild-to-moderate injection site pain, fatigue, and headache. No myocarditis, thrombosis or other vaccine-related safety concerns were identified. A total of 5 study participants in the vaccine group reported pregnancy during the study period with no adverse outcomes being reported. At the time of the pe-specified interim analysis, in the combined per-protocol plus nuclear antibody positive populations, 96 infections had occurred at least 14 days after the second dose, with 52/9246 (0.56%) in the SpikoGen® group and 44/3014 (1.46%) in the placebo group, translating to a vaccine efficacy (VE) against infection of 64.36% [95% CI 46.54 to 76.11]. In the per-protocol participants (serum nuclear antibody negative at baseline), infection occurred in 50/8100 (0.62%) of the SpikoGen® group and 37/2512 (1.47%) in the placebo group for a VE of 59.69%, [95% CI; 37.95 to 73.57]. This exceeded the benchmark minimum criteria set by the WHO which stipulated a clear demonstration of efficacy, ideally with a VE point estimate around 50% and the lower bounds of the 95% confidence interval exceeding 30%.[55] The final trial design was performed in consultation with the Iranian FDA who did not request inclusion of an adjusted confidence interval for VE according to the alpha spending function.[56] At the time of the single interim analysis, 96 infections had already occurred, which was 65.3% of the target total, thereby reducing the risk of a type 1 error despite lack of adjustment for this interim analysis. Of 50 trial infection virus isolates sequenced, all were confirmed as the B.1.617.2 (delta) variant. On the basis of this data, SpikoGen® received an emergency use authorization (EUA) from the Iranian FDA on 6 October 2021 for use in prevention of COVID-19 in adults. Following the EUA, a subsequent post-hoc final analysis was performed with censoring of data at 100 days post-randomization. Efficacy against severe COVID-19 disease remained high at 77.5%, although the efficacy against symptomatic infection in the per-protocol population had reduced to 43.99% (95% CI: 30.30–55.00) at the time of this second analysis. This waning over time of vaccine protection against symptomatic infection was also seen with the other COVID-19 vaccines.[57] Other potential confounders may have contributed to the lower VE against symptomatic infection seen at the time of the second posthoc analysis. As other approved vaccines were available in the latter stages of the Phase 3 trial, a higher proportion of participants in the placebo control arm requested unblinding and dropped out to receive other vaccines, thereby creating imbalances between the two groups. We speculate that the use of a saline placebo may have led to a degree of unblinding due to its lack of injection site reactogenicity, thereby enabling participants to distinguish whether they had had a placebo. The Oxford adenoviral vector vaccine trials were amongst the few to use an active vaccine comparator, meningococcal vaccine.[58] Another factor potentially contributing to the lower efficacy on the second post hoc analysis was that VE is liable to be underestimated during periods of extremely high community infection rates[59] when individuals are repeatedly exposed to high levels of virus. Notably, the delta variant infection rates in the SpikoGen® Phase 3 trial were exceptionally high at 313.35 per 1000 person/yr in the placebo group as compared to much lower infection rates reported in Phase 3 studies of the mRNA[60] and adenoviral vector vaccines,[58] which were less than half this rate. Hence, high community infection rates with delta virus could have reduced estimates of VE during SpikoGen's Phase 3 trial, by making vaccine breakthrough infections more likely. As delta was the only strain found present during the conduct of the SpikoGen® Phase 3 trial, data on SpikoGen® efficacy against the ancestral Wuhan and alpha strains were not able to be assessed, although animal challenge data had shown strong protection against the ancestral strains such as Wuhan.[32] Furthermore, algorithms based on spike-binding IgG and virus neutralization antibody levels were used to predict SpikoGen® efficacy against ancestral strains.[61] These predicted SpikoGen's efficacy at protecting against the ancestral strain in the range of 85–95%[18] Elderly subjects were excluded from the Phase 3 trial as the IFDA did not agree to enrolling participants older than 50 years into a placebo-controlled study at a time when other COVID-19 vaccines were becoming available. This meant that no direct efficacy data is available for SpikoGen® in the elderly. However, SpikoGen® trials that have included older subjects have found no significant correlation between age and serum neutralizing antibody levels (manuscript in preparation). This suggests VE should be preserved in the elderly, even although direct data on this are not available.

Study limitations

It would be particularly interesting to have trial data comparing SpikoGen® vaccine with other protein-based vaccines such as those from Novavax from the United States, Sanofi in France and ZF2001 RBD vaccine from China. Unfortunately, it has not been possible to collect real-world data on SpikoGen's effectiveness outside of clinical trials, due to the challenges of collecting population data in Iran, the only country where SpikoGen has been in widespread population use. It is not possible to directly compare SpikoGen's efficacy

results with those from other vaccine trials, given differences in populations, endpoints, and circulating virus strains, amongst other differences. Notably, SpikoGen® vaccine efficacy was measured against a major wave of delta variant in the Middle East whereas the Phase 3 trials for the mRNA and adenoviral vector vaccines were mostly conducted prior to arrival of the delta variant. Almost all vaccines showed reduced effectiveness against the delta variant as compared to ancestral strains. For example, a test negative case-control study showed 2-dose effectiveness of AstraZeneca adenoviral vaccine to be only 59.8% against B.1.617.2 (delta) versus 66.1% for the earlier B.1.1.7 (alpha) strain.[62] Similarly, a US Veterans Affairs study showed VE against delta of just 13.1% for the Janssen single-dose vaccine, 43.3% for Pfizer, and 58% for Moderna, versus effectiveness against earlier strains (predominantly alpha) of 86.4%, 86.9% and 89.2%, respectively.[62]

A primary course of SpikoGen® vaccine initially comprised two doses given 3 weeks apart. In retrospect, even greater protection might have been achieved with a 3-dose course. This was shown in a recent NHP study where three doses of SpikoGen® vaccine protected against a widely heterologous Omicron BA.5 infection. At the time of the SpikoGen® phase 3 trial, regulatory bodies including WHO had published target product profiles for COVID-19 vaccines that specified a maximum of two doses as the minimal acceptable criteria. This created a major bias against protein-based vaccines as it is not unusual for three or even more primary doses of protein vaccines to be needed to establish maximal protection. For example, pediatric hepatitis B or hemophilus influenza B vaccines recommend three or four doses as the primary course.[63] In retrospect, these minimum acceptable criteria for COVID-19 were overly restrictive, as protection against infection by two doses of any of the COVID-19 vaccines, including adenovirus and mRNA vaccines, waned rapidly and was inadequate to protect against the newly emerging variants such as Omicron. This led to the introduction of 3rd and then 4th dose boosters within months of each other to try and restore vaccine protection against the Omicron strains. Given this extremely short-time interval between doses, these 3rd and 4th doses should in retrospect be considered part of a primary series, meaning that none of the vaccines that received EUA's would have met WHO's minimum acceptable criteria. This is an important point as it would be a grave mistake if similar restrictive minimum acceptability criteria were set for future pandemic vaccines.

Booster doses

A 3rd dose booster study was undertaken with SpikoGen® vaccine in adult subjects aged 18–90 years who had received a primary immunization with a primary course of any COVID-19 vaccine, at least four or more months previously (clinicaltrials.com; NCT05175625). This double-blind and randomized placebo-controlled study was initiated in December 2021 and enrolled 300 previously immunized adult participants, drawn from groups who had previously received either inactivated whole virus, viral vector or recombinant protein (SpikoGen®) COVID-19 vaccines. Participants were randomized on a 3:1 basis to receive a single intramuscular booster dose of SpikoGen® vaccine or saline and then had serum antibody levels measured 14 days after the booster dose. Interestingly, despite the participants having been vaccinated just 4 or more months previously, regardless of what type of vaccine they had received, ~50% already had very low spike antibody levels consistent with waning immunity.[64] In response to the SpikoGen® booster dose, 76% of participants seroconverted by surrogate virus neutralization test (sVNT) against the ancestral Wuhan strain, versus just 3% of the placebo group, where seroconversion was defined as a change in the status of antibody levels from negative to positive based on the prespecified commercial ELISA kit threshold. The inactivated virus-primed group had the highest 25.8-fold rise in sVNT with a 14.3-fold rise in the viral vector-primed group and an 11.3-fold rise in the SpikoGen®-primed group. There were no significant differences in mean sVNT titers between the different primary vaccine groups post the booster dose. Notably, the SpikoGen® booster dose induced broad cross-neutralization of the major SARS-CoV-2 variants of concern, including a 13- to 18-fold rise in serum neutralization of the Omicron BA.1 variant. This indicated a 3-dose primary course of SpikoGen® was needed for optimal for maximum protection against Omicron strains. Just why a 3rd SpikoGen® dose was so critical to the cross-neutralizing antibody response is not known, but similar effects of broader responses after a 3rd dose were reported for other vaccines, suggesting a generalized phenomena.[65] We theorize that three rounds of vaccine stimulation are required to generate immune responses against subdominant epitopes in the spike protein that are highly conserved amongst the variants. This would then explain why three doses of COVID-19 vaccine are so important to get broad cross-neutralizing responses.

The SpikoGen® booster dose was licensed by the Iranian FDA in January 2022. The optimal timing of the 3rd dose is still not known, particularly for new vaccine-resistant variants such as Omicron, to which vaccine immunity wanes faster than against the ancestral strains.[66] A recently completed Australian clinical trial tested the effect of different dose intervals and the results are still being analyzed (ClinicalTrials.gov: NCT05279456). As mentioned above, the response to the 3rd dose was particularly robust for those who had had an inactivated vaccine-prime with this group having a 25.8-fold increase in sVNT from baseline levels. Inactivated vaccines typically present only low and variable amounts of spike protein to the immune system due to the low ratio of spike protein to total virus protein content in the inactivated vaccine, and because some cleavage of S1 from S2 occurs during the beta-propiolactone inactivation process.[67] Hence, we hypothesize that in individuals primed with inactivated or adenoviral vector vaccines, exposure to the much larger dose of spike protein in the SpikoGen® 3rd dose may help stimulate a strong recall response in spike-specific memory B cells that were only weakly induced by the primary course of inactivated vaccine. In addition, boosting with a heterologous vaccine often gives a stronger response than boosting with a homologous vaccine.[68] The Advax-CpG adjuvant in the SpikoGen® booster may also contribute to the strong 3rd dose response by stimulating more broadly cross-neutralizing antibodies. For example, mice immunized with inactivated Japanese encephalitis

virus (JEV) adjuvanted with Advax-CpG were cross-protected against lethal infection with West Nile virus infection.[69] Sera from these JEV+Advax-CpG immunized mice was able to cross-neutralize a broad repertoire of flaviviruses, including West Nile virus, Murray Valley encephalitis virus, St Louis encephalitis virus and dengue viruses.[70]

Special populations

Another important population who need protection during a pandemic is immune-suppressed patients. A single-arm trial of SpikoGen® as a booster dose was performed with 43 kidney transplant patients who had had primary vaccination with 2 doses of Sinopharm inactivated COVID-19 vaccine 1–3 months previously.[71] SpikoGen® induced positive humoral and cellular responses regardless of prior seropositive or seronegative status of the kidney transplant patients, with seroconversion rates 30 days after the booster dose of 35.3% to anti-S_1 binding IgG and 29.4% to surrogate neutralizing antibodies. The most common solicited adverse events were injection site pain and fatigue, which were predominantly mild and transient. No serious adverse events were recorded. These results supported the suitability of SpikoGen® for immune suppressed patients.

Another recently completed pediatric clinical trial compared the immunogenicity of SpikoGen® vaccine in children 5 years and older to young adults (ClinicalTrials.gov: NCT05231590). This showed that a half-antigen dose of SpikoGen® vaccine in children aged 5–11 years gave equivalent immunogenicity and safety to a full antigen dose in children aged 12–18 years and young adults. SpikoGen® vaccine was subsequently licensed by the Iranian FDA for use in children 5 years and older in Jan 2022.[72]

Ongoing development of SpikoGen® platform

Since the first launch of SpikoGen® vaccine 8 million doses have been delivered in the Middle East. The clinical trials and ongoing pharmacovigilance have not revealed any unexpected serious vaccine-associated adverse events. In particular, SpikoGen® vaccine has not been associated with myocarditis/pericarditis[73] or central venous thrombosis,[74] as have occurred after other COVID-19 vaccines. For convenience and speed of protection, the initial 2-dose primary course of SpikoGen® was administered just 3 weeks apart, which may not be optimal for maximum vaccine immunogenicity. For example, the adenoviral vector vaccines were shown to be more protective if the second dose was given 12 weeks after the first dose.[75] In retrospect, a 3rd dose could have been built into the primary vaccine course for maximal efficacy against variants. The effect of changing the dosing interval of SpikoGen® is being studied in two Australian clinical trials, the first looking at the effects of varying the dose interval between the first 2 doses from 3 to 6 weeks (Clinicaltrial.gov; NCT05148871). A second trial is comparing administration of the 3rd dose either 1 or 3 months after the 2nd dose (ClinicalTrials.gov; NCT05279456). Yet a further Australian clinical trial is examining the effect of using SpikoGen® as a booster dose for individuals who have received two or more prior doses of any COVID-19 vaccine type, including mRNA or adenoviral vector vaccines (ClinicalTrials.gov: NCT05542862).

Since the initial clinical trials and licensure of SpikoGen® vaccine, SARS-CoV-2 has continued to mutate at a rapid pace, first into the original Omicron BA.1 to BA.5 strains and then into recombinant lineages such as XBB.1 and more recently into variants such as BA.2.86 (Pirola).[76] Each new variant has been more highly transmissible and more vaccine-resistant than the previous generation. Bivalent versions of the mRNA vaccines were released to the market, containing a mix of mRNA encoding for the original Wuhan and BA.1 (Australian market) or BA.5 (US market) spike proteins to try and compensate for poor efficacy of the original mRNA vaccines. While these showed modest short-term efficacy against the homologous BA.4/5 strains, they were not shown to be effective against the XBB lineage.[77] The bivalent mRNA vaccines have already been replaced by monovalent Omicron XBB.1.5 vaccines, although the effectiveness of these against new Pirola subvariants such as JN.1 is unknown.

The continual generation of SARS-CoV-2 immune-escape variants thereby presents an ongoing major challenge for all COVID-19 vaccines. As described above, multivalent (bi-, tri-, quadra- and even penta-valent) formulations of SpikoGen® vaccine that include multiple-spike proteins have been produced and shown immunogenic and protective in animal studies. In addition, updated monovalent SpikoGen® boosters targeting BA.2.86 are currently being developed. Parenterally administered vaccines while effective in preventing lung virus replication and severe disease, have proved less effective at preventing initial infection and have not stopped virus transmission,[78] SpikoGen® is a possible exception, having been shown to block virus transmission in the hamster model.[49] SpikoGen® has recently been tested as a mucosal booster delivered via either oral or intrapulmonary routes in NHP.[50] While nasal administration is another alternative route for mucosal immunization, we chose oral administration because of its convenience (it does not require a specialized delivery device), and because we speculate that priming of gut associated lymphoid tissue (GALT) may be beneficial in control of SARS-CoV-2 infection. The results suggested that the oral booster might indeed provide enhanced protection against a heterologous Omicron BA.5 challenge. The effect of an oral SpikoGen® booster alone or in combination with a quadrivalent seasonal influenza vaccine is to be tested in an Australian clinical trial (clinicaltrial.gov NCT06355232).

Conclusions

The adenoviral vector vaccines have been largely discontinued from use due to major safety concerns including deaths from thromboembolic disease, as well as weak efficacy. While most attention remains on the mRNA vaccines, adjuvanted recombinant protein vaccines such as SpikoGen® offer a suitable alternative, with lower reactogenicity and better safety. As yet, there is very little head-to-head data comparing the efficacy of booster doses of mRNA and recombinant protein COVID-19 vaccines, with most comparison studies just based on serum antibody levels. Notably, absence of serum neutralizing antibody in SpikoGen®-vaccinated animals did

not mean they were not protected.[50] Conversely, we have seen individuals with high serum antibody levels after mRNA vaccination still develop SARS-CoV-2 infection. A Danish registry study concluded "quantitative levels of anti-spike IgG have limited impact on the risk of breakthrough infection with Omicron."[79] Hence, any head-to-head comparative efficacy studies of different COVID-19 vaccines would need to compare clinical endpoints such as disease severity rather than just compare serum antibody surrogates. Furthermore, there is the possibility that the kinetics and patterns of protection may be different between recombinant protein and mRNA vaccines. mRNA vaccines appear to provide a short sharp burst in antibodies with associated short-term reductions in infection risk that then rapidly fade away. By contrast, adjuvanted protein vaccines may provide more durable protection despite inducing lower antibody titers, particularly if they facilitate early mild infections that could then provide more robust hybrid immunity going forward.[80] This could present a paradox where the apparently weaker vaccine that allows an early mild infection might provide better long-term outcomes than a initially more potent vaccine that prevents infection short-term but which leaves the individual vulnerable to repeated infections when the initial protection rapidly wanes. This could explain recent data showing that the more doses of mRNA vaccine an individual received, the greater their risk of experiencing a SARS-CoV-2 infection.[81]

Long-term safety is another consideration when assessing the relative merits of recombinant protein versus mRNA vaccines, as the long-term effects of repeated doses of mRNA remain unknown. By contrast, repeated doses of protein-based vaccines such as influenza vaccines are known to be able to be given annually for many years with no cumulative adverse effects.[82] The last four years have seen a battle between reliable protein-based technologies with well-understood safety and mechanism of action versus newer mRNA and viral vector approaches that continue to have uncertainty around their mechanisms of action and long-term effects. The vaccine industry has traditionally been extremely conservative. In a twist of fate, the inability of large vaccine companies to commit to ultrashort pandemic vaccine delivery timelines demanded by politicians meant that the US WarpSpeed and UK government funding initiatives instead directed vast sums to accelerate development of nascent technologies, in particular mRNA and adenoviral vectors, based on their promises of speedy delivery. Only time will tell whether this was the right move. One might ask whether regulators are now prepared to routinely accept any new vaccine technology with minimal animal testing before permitting its use in billions of people or whether this was just a pandemic anomaly.[73] We still do not know the significance of mRNA vaccines increasing IgG4 levels[83] and what might this mean for the effectiveness of mRNA in the longer-term.[84] Only time will ultimately tell. SpikoGen® represented a remarkable achievement given the extremely limited resources available to the project team. SpikoGen® may be the only COVID-19 vaccine that was successfully developed without major government or instirutional support evertheless, it was delivered in world record time for a recombinant protein vaccine. Traditional protein-based vaccines have stood the test of time and have saved countless hundreds of millions of lives. Whether mRNA vaccines can achieve the same going forward is still not clear. In the meantime, it would be wise for funders to continue to support protein-based vaccine development and not neglect this area due to excess mRNA vaccine euphoria.

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Disclosure statement

The author is affiliated with Vaxine Pty Ltd which holds the rights to COVAX-19®/Spikogen® vaccine and Advax™ and CpG55.2™ adjuvants. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Institutional Review Board Statement

All described clinical studies were conducted according to the guidelines of the Declaration of Helsinki and approved by the appropriate Institutional Review Boards. Informed consent was obtained from all subjects involved in clinical studies.

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