Scalable live-attenuated SARS-CoV-2 vaccine candidate demonstrates preclinical safety and efficacy

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Successfully combating the COVID-19 pandemic depends on mass vaccination with suitable vaccines to achieve herd immunity. Here, we describe COVI-VAC, the only live attenuated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccine currently in clinical development. COVI-VAC was developed by recoding a segment of the viral spike protein with synonymous suboptimal codon pairs (codon-pair deoptimization), thereby introducing 283 silent (point) mutations. In addition, the furin cleavage site within the spike protein was deleted from the viral genome for added safety of the vaccine strain. Except for the furin cleavage site deletion, the COVI-VAC and parental SARS-CoV-2 amino acid sequences are identical, ensuring that all viral proteins can engage with the host immune system of vaccine recipients. COVI-VAC was temperature sensitive in vitro yet grew robustly (>107 plaque forming units/ml) at the permissive temperature. Tissue viral loads were consistently lower, lung pathology milder, and weight loss reduced in Syrian golden hamsters (Mesocricetus auratus) vaccinated intranasally with COVI-VAC compared to those inoculated with wild-type (WT) virus. COVI-VAC inoculation generated spike IgG antibody levels and plaque reduction neutralization titers similar to those in hamsters inoculated with WT virus. Upon challenge with WT virus, COVI-VAC vaccination reduced lung challenge viral titers, resulted in undetectable virus in the brain, and protected hamsters from almost all SARS-CoV-2-associated weight loss. Highly attenuated COVI-VAC is protective at a single intranasal dose in a relevant in vivo model. This, coupled with its large-scale manufacturing potential, supports its potential use in mass vaccination programs.

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This work demonstrates the feasibility of rationally designing and synthesizing vaccine candidates for testing in response to an emerging disease in real world conditions. Furthermore, using a live attenuated codon-pair-deoptimized virus approach ensures that all components of the host immune system will be engaged, and potential effects from the vector sequences from hybrid live viruses are avoided. Evidence from other codon-deoptimized viruses suggests that COVI-VAC will be resistant to reversion and loss of potency due to antigenic drift. The ease of large-scale virus growth under permissive conditions coupled with the potential for single-dose intranasal administration make COVI-VAC an appealing candidate for clinical testing for possible use in mass immunization programs.

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remains unchanged, and the codon usage of the ORF is also maintained.

In our COVI-VAC, the coding region for the spike protein was recoded. Notably, except for the deleted furin cleavage site, the codon-pair–deoptimized COVI-VAC genome encodes the exact same proteins (same amino acid sequences), and uses the same codons, as the original (WT) virus. Yet, the recoding in COVI-VAC leads to 283 point mutations in the viral genomic sequence, and the virus variant is highly attenuated. Because the amino acid sequence is a perfect match to that of the circulating WT strain, every viral antigen is present, providing the potential for a broad immune response and making the vaccine more likely to retain efficacy if there is genetic drift (3).

Because SAVE vaccine attenuation results from hundreds or thousands of mutations, selective pressure on a particular mutation to revert to WT appears to be reduced (10–14). Deep sequencing of two lines of a SAVE respiratory syncytial virus (RSV) LAV candidate containing 2,692 point mutations revealed only low-level sporadic mutations after 18 passages at the permissive temperature (14). A second RSV strain containing 1,578 point mutations, was genetically stable for at least eight passages at the permissive temperature with only sporadic, low-level mutations found in 56 deep sequencing and 8 Sanger sequencing (14, 15). Thus, the myriad of mutations that contribute to the attenuated phenotype of SAVE-LAVs all appear to confer high stability with respect to reversion to pathogenicity (10–14).

SAVE technology can be applied to any virus that is susceptible to reverse genetics techniques (16–19). First developed in the poliovirus system (7), SAVE has been extended to influenza virus, RSV, dengue virus, and Zika virus, among others (16, 17). It can be employed to design and synthesize multiple vaccine candidates from the viral genome sequence alone, or as described here, from the intact virus using a reverse transcription and recovery process. We describe here the SAVE design, assembly, and preclinical assessment of a SARS-CoV-2 LAV candidate, COVI-VAC.

Results

We applied SAVE technology to recover and amplify WT coronavirus strain 2019-nCoV/USA-WA1/2020 (WA1) (distributed by BEI Resources, GenBank accession No. MN985325) and rationally designed two deoptimized LAV candidates against the strain (20). The WT WA1 virus was deposited by the US Centers for Disease Control and Prevention (CDC) and was obtained from BEI Resources. Its sequence was verified by us (20). A schematic of the SARS-CoV-2 genome is shown in Fig. 1A. To construct two LAV candidates, a portion of the genome-encoding spike gene sequences (Fig. 1A) was replaced by the corresponding codon-pair–deoptimized spike gene sequences. COVI-VAC (strain CDX-005) contains 283, whereas strain CDX-007 contains 149 silent mutations relative to WT WA1 virus. The full-length WT WA1 and deoptimized complementary DNAs were transcribed in vitro to make full-length viral RNA that was electroporated into Vero E6 cells. Transfected cells were incubated for 6 d or until a cytopathic effect (CPE) appeared. Infection medium was collected on days 2, 4, and 6. Virus titer was determined by plaque assay on Vero E6 cells. Transfected cells were incubated for 6 d or until a cytopathic effect appeared. Infection medium was collected on days 2, 4, and 6. Virus titer was determined by plaque assay on Vero E6 cells. Transfected cells were incubated for 6 d or until a cytopathic effect appeared. Infection medium was collected on days 2, 4, and 6.

The reduced growth at 37 °C adds another layer of in vivo safety, sooner for cultures grown at 37 °C. In contrast, COVI-VAC grew more slowly at 37 °C than at 33 °C. The robust growth at 33 °C ensures that COVI-VAC can be easily scaled for mass production, while the reduced growth at 37 °C adds another layer of in vivo safety to the potential vaccine.

To investigate the in vivo properties of COVI-VAC, we turned to Syrian golden hamsters (Mesocricetus auratus). A recent survey of animal models indicates that these hamsters recapitulate many of the characteristics of human COVID-19 disease and are a relevant small animal model for COVID-19 (26). SARS-CoV-2 replicates efficiently in hamster lungs, causing severe pathological lesions following intranasal infection (27). As has been published, viral antigens are present in nasal mucosa, bronchial epithelium, and areas of lung consolidation on days 2 and 5 after SARS-CoV-2 inoculation, and they are cleared on day 7 (28). Clinical signs included rapid breathing, weight loss, histopathological changes in the lung/airway, intestinal involvement, spleen and lymphoid atrophy, and cytokine activation within 1 wk of virus challenge (29). Infected hamsters can infect other hamsters housed in the same cage, and neutralizing antibodies (Abs) are detected on day 14 postchallenge (29).

We evaluated the attenuation, safety, and efficacy of COVI-VAC in 5- to 6-wk-old hamsters under BSL-3. hamsters were dosed IN on day 0 with 0.05 mL of either 5 × 10^4 or 5 × 10^5 PFU/mL WT WA1 or 5 × 10^5 PFU/mL COVI-VAC. Because COVID-19 disease is associated with pulmonary, olfactory, and neural dysfunction (30–32), we measured viral load in homogenized lungs, olfactory bulbs, and brains. Total viral RNA measured by qPCR was near or below the limit of detection in lung, olfactory bulb, and brain on days 2 and 4 postinoculation (PI) in COVI-VAC–inoculated hamsters. In contrast, viral RNA was detected in WT WA1–infected hamsters in all three tissues at both times (Fig. 2A–C). To investigate the infective virus was present, we performed tissue culture infective dose 50 (TCID50) assays on lung homogenates on days 2, 4, and 6 PI (Fig. 3). By day 4, infectious virus loads were below the limit of detection (<32 TCID50/mL) in the lungs of animals inoculated with COVI-VAC, whereas they remained greater than 10^2 and 10^2 TCID50/mL in animals infected with WT WA1 at 5 × 10^3 and 5 × 10^3 PFU, respectively (n = 3/group; P < 0.01 by factorial ANOVA for independent samples). Thus, COVI-VAC is highly attenuated relative to WT WA1 in vivo.

To evaluate the safety of COVI-VAC, the change in weight in hamsters was monitored for 9 d after inoculation. Hamsters inoculated with COVI-VAC experienced weight gain during the period, whereas those inoculated with WT WA1 at either dose experienced weight loss (Fig. 4A). Only at day 9 PI did any WT WA1–infected animals return to their starting weight (Fig. 4A). A mixed model ANOVA indicated that the changes were significantly different between COVI-VAC– and WT WA1–treated groups (P < 0.001).

We also performed histological examinations of the lungs, brain, and kidney. Formalin-fixed paraffin sections were stained with hematoxylin and cosin, and light microscopic evaluation was conducted by a blinded board-certified veterinary pathologist (n = 3 per group). Multiple parameters were scored on a 0 to 5 pathology rating scale. No changes were noted in brain and kidney sections of hamsters administered WT WA1 or COVI-VAC. That is, the histopathology scores for all animals in all groups were zero. Consistent with the pathological cellular infiltration found in lungs...
of humans with COVID-19, however, alveolar and/or perivascular or peribronchiolar mixed cell infiltrates, necrosis of the bronchiolar or bronchial epithelium with neutrophilic infiltration into the lumen, and perivascular edema occasionally accompanied by hyperplasia of the bronchiolar or bronchial epithelium was seen in hamsters infected with WT WA1 (Fig. 4B and C). In contrast, lung pathology was limited to minimal alveolar to mild perivascular or peribronchiolar mixed cell infiltrates at day 6 PI in COVI-VAC–inoculated hamsters (Fig. 4B and C).

To assess efficacy of COVI-VAC as a vaccine, we measured its ability to induce Abs against WT WA1. First, we performed an enzyme-linked immunosorbent assay (ELISA) to determine IgG titers against SARS-CoV-2 spike S1 in control sera and sera of hamsters inoculated with WT WA1 or COVI-VAC. Like WT WA1 infection, COVI-VAC inoculation induced a strong anti-spike S1 Abs response (Fig. 5A). We then tested for the presence of neutralizing Abs against WT WA1 virus in serum of inoculated hamsters by plaque reduction neutralization titer (PRNT) on day 16 PI. We calculated 50, 80, and 90% neutralization (inhibition of plaque formation) with serum dilutions up to 1,280-fold. All COVI-VAC–inoculated hamsters produced neutralizing Ab titers at levels similar to those induced by WT WA1 inoculation (Fig. 5B).

We measured the efficacy of COVI-VAC in two challenge studies. In the first study, hamsters were vaccinated IN with a single dose of $5 \times 10^4$ PFU COVI-VAC and then challenged IN with $5 \times 10^4$ PFU WT WA1 on day 16 PI. Lungs were harvested on day 18 (day 2 postchallenge) and viral loads measured by qRT-PCR. Viral loads of the challenge WT WA1 virus were reduced by more than 50,000-fold on average in the lungs of COVI-VAC–vaccinated compared to unvaccinated hamsters, attesting to the efficacy of the vaccine (Fig. 6A). Whereas SARS-CoV-2 virus was detected in the brains of challenged naive hamsters, none was detected in

![Fig. 1. Growth of deoptimized SARS-CoV-2 vaccine candidates. (A) Schematic presentation of the SARS-COV-2 viral RNA genome (29,674 nucleotides) and the viral polyprotein, indicating regions encoding numerous viral proteins. Also shown in detail is the coding segment S, the portion of the polyprotein that encodes the spike protein. SP = signal protein; NTD = N-terminal domain RBD = receptor binding domain; FP = fusion peptide; HR1 = heptad repeat 1; HR2 = heptad repeat 2; and TM = transmembrane domain. Indicated in spike Inset is the cleavage region that includes the S1/S2 cleavage site that contains the furin cleavage site that was deleted in COVI-VAC and the S' cleavage site. (B) Plaque phenotypes of natural and synthetic WT SARS-CoV-2 and deoptimized SARS-CoV-2 viruses COVI-VAC and CDX-007 after 67-h incubation in Vero E6 cells. (C) Vero cells were infected with WT WA1 or COVI-VAC at 33 or 37 °C. Infected wells were harvested at the indicated time points, and titers were determined by plaque forming assays and reported as log of PFU/mL culture medium.](https://doi.org/10.1073/pnas.2102775118)

![Fig. 2. In vivo attenuation of COVI-VAC in hamsters. Hamsters were inoculated with $5 \times 10^6$ or $5 \times 10^5$ PFU of WT WA1 or $5 \times 10^4$ PFU COVI-VAC. Viral RNA was measured by qPCR at days 2 and 4 PI in the (A) olfactory bulb, (B) brain, and (C) lungs. (n = 3/group; Bars = SEM).](https://doi.org/10.1073/pnas.2102775118)
and challenged with 5 × 10^4 or 5 × 10^5 PFU of WT WA1 or 5 × 10^4 PFU COVI-VAC. Infectious viral load in lung tissue was assessed by TCID_{50} assay and expressed as \log_{10} of TCID_{50}/mL. Differences between COVI-VAC and WT WA1–treated groups were significant (n = 3/group; P < 0.01 on days 4 and 6; Bars = SEM).

In the second challenge study, hamsters were inoculated as above and challenged with 5 × 10^4 PFU WT WA1 on day 27 postvaccination. Lungs were harvested on day 29 or 31 (day 2 or 4 postchallenge) and viral loads measured by qRT-PCR. Again, viral loads were significantly reduced by >10^4-fold at both days (Fig. 6B). Efficacy was also evaluated in these animals by measuring the ability of COVI-VAC to protect against SARS-CoV-2–induced weight loss (Fig. 6C). Animals inoculated with vehicle exhibited significant weight loss by day 2 postchallenge and continued to lose more weight through at least day 5 postchallenge. In contrast, hamsters inoculated with either WT WA1 or COVI-VAC showed no significant weight loss during the same period. We also assessed neutralizing antibody titers on day 27, just prior to challenge. Microneutralization tests showed that COVI-VAC–immunized animals had >700-fold higher SARS-CoV-2 virus neutralizing antibody titers than at baseline and >2-fold higher than hamsters previously inoculated with WT WA1 virus.

Discussion

We generated a synthetic highly attenuated live vaccine candidate, COVI-VAC, by recoding portions of the WT SARS-CoV-2 genome according to the SAVE algorithm of codon-pair bias deoptimization (7). We suggest that a variety of different mechanisms may collaborate in the attenuation by codon-pair deoptimization: 1) reduced rate of translation, 2) protein misfolding, 3) changes in messenger RNA (mRNA) secondary structures, 4) altered regulatory signals, and 5) increased numbers of 3′-Cp5′-G or 3′-Up5′-A dinucleotides linking two codons. One or all of these processes may contribute to an impairment of genome replication (9). Moreover, many codon-pair–deoptimized viral genomes acquire a temperature sensitive phenotype at 37 °C (Fig. 1C) that by itself can be a major contributor to restrict virus proliferation in vivo. Whatever the mechanism, the attenuated COVI-VAC virus presents every viral antigen, providing the potential for a broad immune response and making it likely to retain efficacy even if there is genetic drift in the target strain (3). Also, as with other SAVE vaccines, COVI-VAC is expected to be highly resistant to reversion to pathogenicity since hundreds of silent (synonymous) mutations contribute to the phenotype. Our preliminary tests of reversion suggest that the vaccine is stable as assessed by bulk sequencing of late passage virus.

The unique polybasic furin cleavage site present in the WT SARS-CoV-2 spike protein is emerging as a key driver of the lower respiratory tract tropism of the virus and the resulting pathology of COVID-19. While a functional furin cleavage site is not required for efficient SARS-CoV-2 growth in Vero cells, the furin proteolytic processing is critical for efficient infection of TMRPSS2-expressing cells abundant in the lower respiratory tract (24). Further, furin processing of the spike protein may expand the tropism of SARS-CoV-2 to the central nervous system via interaction with neuropilin-1 (33, 34). Finally, the furin cleavage of spike has been suggested to aid SARS-CoV-2 in evading the host innate interferon response (25). Consequently, SARS-CoV-2 viruses without an active furin cleavage site have been shown to be attenuated in small animal models (22, 23) and display reduced capacity of host transmission (35). For these reasons we chose to eliminate the furin cleavage site in the spike protein of COVI-VAC, as a second and independent determinant of its attenuation. While the overall attenuation phenotype of the COVI-VAC strain is likely a combination of both determinants (codon-pair deoptimization and furin cleavage site deletion), we currently cannot say how much either determinant contributes individually.

Like codon-pair deoptimization, the deletion of the furin cleavage is expected to provide a very stable genetic attenuation marker. After 20 passages of COVI-VAC in Vero manufacturing cells, there was no evidence of restoration of the deleted furin cleavage site.

Our hamster studies demonstrate that COVI-VAC is highly attenuated and safe in these animals. It induced lower total viral...
loads in the lungs and olfactory bulb as well as lower live viral loads in the lung of animals inoculated with COVI-VAC compared to WT WA1. Virus was not detected in brains of COVI-VAC-inoculated hamsters, and unlike WT virus, COVI-VAC inoculation did not induce weight loss or significant lung pathology in infected hamsters.

The hamster studies also suggest that an episode of COVI-VAC infection may effectively protect against illness with SARS-CoV-2. Assessment of Abs titers demonstrate that it is as effective as WT virus in inducing serum IgG and neutralizing Abs. It is protective against WT challenge: inoculation with COVI-VAC leads to lower lung viral titers and complete protection against virus in the brain. Hamsters inoculated with COVI-VAC also do not exhibit the weight loss observed in vehicle-inoculated animals. Moreover, there is no evidence of disease enhancement.

Work remains to be done on characterizing the immune response elicited by COVI-VAC. Studies described here were limited by the capabilities of BSL3 laboratories and the paucity of hamster-specific reagents available early in the pandemic, when this study was conducted. Our current work and the analyses of a Phase 1 clinical trial (ClinicalTrials.gov Identifier: NCT04619628) will shed more light on the profile of the immune response following COVI-VAC vaccination. Regardless of the mechanism of protection, the data presented here demonstrate that, in animals, a single dose of COVI-VAC protects against a WT challenge and promotes the formation of a virus-neutralizing antibody response as potent as that resulting from a WT infection [or possibly more so (see Fig. 6D)].

Together, our data suggest that COVI-VAC may be part of a potentially important class of LAVs currently being developed for use in animals and humans (14, 17, 18, 36–38). Like other codon-pair–deoptimized vaccines, it presents all viral antigens with their native amino acid sequence, can be administered IN, is safe and effective in small animal models with a single dose, appears to be resistant to reversion, and can be grown to high titers at a permissive temperature. A Phase 1 clinical trial is currently underway to test its safety and efficacy in humans.

Materials and Methods

Virus Production. LAV candidates against SARS-CoV-2 were generated by recovering viral genomes from WT SARS-CoV-2, strain USA-WA1/2020 (GenBank accession no. EU908380.1). The LAV candidates against SARS-CoV-2 were propagated in HEK293 cells and were harvested at approximately day 3 posttransfection.

Assay of infectivity. HEK293 cells were seeded in a 96-well plate at 10,000 cells/well. After 24 h, the cells were infected with COVI-VAC or vehicle at a multiplicity of infection of 0.001 (MOI). The infection was allowed to proceed for 1 h, and then the virus-containing medium was removed, and fresh medium was added. The plates were incubated for 48 h at 37°C. The viral infectivity was determined by plaque assay and plaque reduction neutralization test (PRNT). The PRNT is the reciprocal of the last serum dilution that reduced plaque numbers 50, 80, or 90% relative to those in wells containing serum from preimmune hamsters. Titer for vehicle were <eightfold dilution and are plotted as 5 on the graph. (n = 3/group; P < 0.05 for all groups versus vehicle; Bars = SEM).

Assay of immune response. The hamster studies also suggest that an episode of COVI-VAC infection may effectively protect against illness with SARS-CoV-2. Assessment of Abs titers demonstrate that it is as effective as WT virus in inducing serum IgG and neutralizing Abs. It is protective against WT challenge: inoculation with COVI-VAC leads to lower lung viral titers and complete protection against virus in the brain. Hamsters inoculated with COVI-VAC also do not exhibit the weight loss observed in vehicle-inoculated animals. Moreover, there is no evidence of disease enhancement.

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accession No. MN988325). The reagent was deposited by the Centers for Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH, and ATCC (ATCC-T923, ATCC-PMR1122, and ATCC-NR-52285). To construct two LAV candidates, a portion of the genome-encoding spike gene sequences was replaced by the corresponding codon-pair-deoptimized spike gene sequences (7). COVI-VAC contains 283 and CDX-007 contains 149 silent mutations relative to WT WA1 virus.

Transfection of Vero E6 cells by RNA electroporation. Full-length RNA transcripts of the genomes were in vitro transcribed and electroporated into Vero E6 cells to recover live virus. Vero E6 cells were obtained from the American Type Culture Collection (CRL-1586™) and maintained in high glucose Dulbecco’s modified Eagle medium (DMEM)/10% fetal bovine serum (FBS). Cells were electroporated with 10 μg purified full-length viral RNA transcripts and 5 μg capped WA1-N mRNA using the MaxCyte ATX System (MaxCyte) according to manufacturer’s instructions. Briefly, 3 × 10^4 Vero E6 cells were once washed in MaxCyte electroporation buffer, resuspended in 100 μL of the same, mixed gently with the RNA, and transferred to MaxCyte OC-100 processing assemblies. Electroporation was performed using the preprogrammed Vero cell electroporation protocol. After 30-min recovery of the transfected cells at 37 °C/5% CO2, cells were resuspended in warm DMEM/10% FBS, seeded at different densities (1/2, 1/3, or 1/6 of the total cells), then incubated at 37 °C/5% CO2 for 6 h or until CPE appeared. Infection medium was collected with complete media change at day 2 and day 4 (DMEM/5%FBS). Virus was detectable by plaque assay as early as 2 d post-transfection, with peak virus generation between days 4 to 6.

Passaging of stock virus. Vero E6 cells were cultured in T25 flasks to near confluency. Prior to infection, spent cell culture medium was replaced with 2 mL fresh DMEM without FBS. A total of 100 μL supernatant from infected flask were added and gently mixed. After a 1-h incubation at 37 °C/5% CO2, inoculum was discarded, and fresh complete medium was added. Cells were incubated until CPE was visible.

Virus Characterization. Plaque titration of SARS-CoV-2 in Vero E6 cells. Serial 10-fold dilutions were prepared in DMEM/2%FBS. A total of 0.5 mL of each dilution was added to 12 wells of 80% confluent Vero E6 cells. After 1-h incubation at 37 °C, the inoculum containing 0.5 mL of semi-saturated Vero E6 cells, infected with 50 μL of WT WA1 SARS-CoV-2 or COVI-VAC. All animals were observed twice (day 0 to 8; day 27 to 31) or once (day 9 to 26) daily. In total, 40 × 10^4 PFU COVI-VAC, 40 × 10^4 PFU WT WA1, and 12 × 10^4 PFU WT WA1 were weighed daily for up to 9 d. The N decreased over time for each group as animals were euthanized for other endpoints on various days PI. The minimum N for 5 × 10^4 PFU COVI-VAC and 5 × 10^4 PFU WT WA1 was 10 and for 5 × 10^4 PFU WT WA1.

On day 27, sera were collected from the remaining hamsters. Hamsters were then challenged on day 27. Weights were recorded daily from day 27 (day 0 PC) through day 31 (day 5 PC). By day 28, only five to six hamsters per group remained, due to scheduled tissue sampling throughout the study. For PC weights, n = 5 to 6 at day –1 to 3 and n = 3 at day 4 to 5 PC.

Tissue harvesting. On days 2, 4, and 6 PI, three hamsters from each group and three hamsters on day 18 from animals challenged on day 16 were euthanized by intravenous injection of Beuthanasia at 150 mg/kg. The left lung was collected for viral load determination. To measure viral load, lung was homogenized in a 10% w/v in DMEM with antibiotics using a bead mill homogenizer (Omni International). Infectious virus titers were determined by TCID50 assay titrating 10-fold serial dilutions of the lung homogenate on Vero E6 cells and are expressed in log10 TCID50 units/mL. RNA was extracted from 100 μL of brain homogenate using a QIA Viral Kit (Zymo Research) according to the manufacturer’s protocol. qRT-PCR was performed using the iQ5 one-step universal probe kit (Bio-Rad) using the following PCR cycling conditions: 40 cycles of 1 s at 95 °C, 3 s at 60 °C, and 20 s at 72 °C.

Antibodies. Plaque reduction neutralization titer. Hamster sera collected at day 16 PI were heat inactivated for 30 at 56 °C. Two-fold serial dilutions in 50 μL DMEM/1% FBS were performed in 96-well plates, and –30 PFU of WA1 in 50 μL DMEM/1% FBS was added to each to a final volume of 100 μL with a total initial serum dilution of 1:10. After incubation at 37 °C/5% CO2 for 1 h, the dilutions were added to confluent monolayers of Vero E6 cells (seeded 1 d prior in DMEM/5%FBS in 24-well plates) with 150 μL fresh DMEM/1% FBS. After 1 h virus adsorption at 37 °C/5% CO2, 750 μL semi-solid overlay was added to the 24-well plates for a final concentration of 1x DMEM, 1.75% FBS, 1% penicillin, 0.5% streptomycin, and 0.05% gentamicin/mL. Plates were incubated until a total volume of 1 mL. Plates were incubated 48 h at 37 °C for plaque formation. Plaques were visualized using 1% crystal violet in 50% methanol/4% formaldehyde. The PNT 50, 80, and 90 were determined as the reciprocal of the last serum dilution that reduced plaque numbers by 50%, 80%, or 90%, respectively, relative to the plaques in nonneutralized wells containing naive hamster serum. Sera that failed to neutralize at the lowest dilution (80%) were assigned a titer of 5, and sera that neutralized >90% at the highest tested serum dilution (1:280) were assigned a titer of ≥1:280.

Antibodies. IgG ELISA. The 96-well plates were coated with SARS-CoV-2 (2019-nCoV) spike S1-His (Sino Biological) at 30 ng/well in 50 μL PBS/0.05M carbonate/bicarbonate buffer pH 9.6 overnight at 4 °C. Plates were blocked with 10% goat serum in PBS 2 h at 37 °C, washed 4x with washing buffer (0.1% Tween 20 in PBS), then incubated with a serially diluted serum (1:10 starting dilution and twofold thereafter) in 10% goat serum/0.05% Tween-20 in PBS and incubated 1 h at 37 °C. Plates were washed 4x with washing buffer then incubated with 1:10,000 horseradish peroxidase–conjugated affinity pure goat anti-Syrian hamster IgG (H & L) (Jackson ImmunoResearch Laboratories) for 1 h at 37 °C. Plates were then washed 4x with washing buffer, and o-phenylenediamine dihydrochloride (Thermo Scientific) was added to the wells and plates were incubated in the dark at 25 °C for 10 min. Reactions were stopped with 50 μL 2.5M sulfuric acid solution and the absorbance at 490 nm read. Relative IgG levels were reported as the log of the dilution at which the absorbance reached 5× above the no serum control background.

Antibodies. Microneutralization. D27 sera were heat inactivated 5 min at 56 °C, then a series of twofold dilutions in DMEM (Gibco11965)2% heat-inactivated serum (Gibco15310) was prepared. Control sera were diluted identically. Diluted sera in a final volume 120 and 60 μL of 2,000 TCID50/mL SARS-CoV-2 (Southern Research) were plated in 96-well trays. Plates were sealed and incubated at 37 °C for 2.5 h. Medium was removed from wells of a 96-well tray that had
been seeded with 2 × 10^4 Vero E6 cells/well the day before, and 100 μL of each serum/SARS-CoV-2 virus mixture added to each of two wells. Plates were covered and incubated 96 h at 37 °C with 5% CO2. Plates were removed from incubator and rested at room temperature for 20 to 40 min, then 100 μL of CellTiter-Glo (Promega) prepared according to manufacturer's instructions was added to all wells, and the plates were gently tapped to mix contents. Plates were incubated 20 min and then read for luminescence.

Data Availability. COVI-VAC is available for distribution to entities that sign a formal material transfer agreement with Codagenix Inc. COVI-VAC sequence is available on GenBank with accession number MZ2404503. The algorithm used to generate the deoptimized viruses was published with the initial provisional US patent application 61/068,666 that can be accessed at “PublicPAIR” (https://portal.uspto.gov/pair/PublicPAIR).

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