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Antibody signature induced by SARS-CoV-2 spike protein immunogens in rabbits

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Multiple vaccine candidates against SARS-CoV-2 based on viral spike protein are under development. However, there is limited information on the quality of antibody responses generated with these vaccine modalities. To better understand antibody responses induced by spike protein–based vaccines, we performed a qualitative study by immunizing rabbits with various SARS-CoV-2 spike protein antigens: S ectodomain (S1+S2; amino acids 16 to 1213), which lacks the cytoplasmic and transmembrane domains (CT-TM), the S1 domain (amino acids 16 to 685), the receptor binding domain (RBD) (amino acids 319 to 541), and the S2 domain (amino acids 686 to 1213, lacking the RBD, as control). Resulting antibody quality and function were analyzed by enzyme-linked immunosorbent assay (ELISA), RBD competition assay, surface plasmon resonance (SPR) against different spike proteins in native conformation, and neutralization assays. All three antigens (S1+S2 ectodomain, S1 domain, and RBD), but not S2, generated strong neutralizing antibodies against SARS-CoV-2. Vaccination-induced antibody repertoire was analyzed by SARS-CoV-2 spike genome fragment phage display libraries (SARS-CoV-2 GFPDL), which identified immunodominant epitopes in the S1, S1-RBD, and S2 domains. Furthermore, these analyses demonstrated that the RBD immunogen elicited a higher antibody titer with five-fold higher affinity antibodies to native spike antigens compared with other spike antigens, and antibody affinity correlated strongly with neutralization titers. These findings may help guide rational vaccine design and facilitate development and evaluation of effective therapeutics and vaccines against COVID-19 disease.

INTRODUCTION

The ongoing pandemic of SARS-CoV-2 has resulted in more than 4.3 million human cases and 290,000 deaths as of 12 May 2020 (1). Therefore, development of effective vaccines for prevention and medical countermeasures for treatment of SARS-CoV-2 infection is a pressing global priority. The spike glycoprotein has been identified as the key target for protective antibodies against both SARS-CoV-1 and SARS-CoV-2 (2–5). Consequently, multiple versions of the SARS-CoV-2 spike proteins are currently under evaluation as vaccine candidates using different modalities and delivery systems (6). However, only limited knowledge exists on antibody repertoire or quality of the immune response generated after vaccination by different spike vaccine antigens (6). Therefore, it is important to perform a comprehensive evaluation of post-vaccination antibody response to elucidate the quality of the immune responses elicited by spike-based vaccine candidates. This could also determine immune markers that may predict clinical benefit that would facilitate evaluation of vaccine candidates.

To better understand vaccination-induced antibody response in a pharmacological/toxicological animal model, we immunized rabbits with several SARS-CoV-2 spike proteins: spike ectodomain (S1+S2), S1 domain, receptor binding domain (RBD), and S2 domain, which lacks the RBD, as a control. Post-vaccination sera were analyzed by genome fragment phage display libraries covering the entire spike gene (SARS-CoV-2 GFPDL) to determine the polyclonal antibody epitope repertoire generated after vaccination as previously applied for other diseases (7–11). In addition, we used several antibody binding assays [enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR)–based real-time kinetics assay] (11–13) and an in vitro SARS-CoV-2 pseudovirion or wild-type virus neutralization assay to measure the quality and function of the antibodies elicited by the different SARS-CoV-2 spike antigens. This study could inform development and evaluation of SARS-CoV-2 vaccines and therapeutics based on the spike glycoprotein.

RESULTS

Rabbit immunization with SARS-CoV-2 spike antigens

Most spike-based vaccines currently under development are designed to contain the RBD (amino acids 319 to 541) in some form. Therefore, we evaluated four different commercially available SARS-CoV-2 spike protein and subdomains: the spike (S1+S2) ectodomain (amino acids 16 to 1213), the S1 domain (amino acids 16 to 685), the RBD domain (amino acids 319 to 541), and the S2 domain (amino acids 686 to 1213), devoid of RBD, as a control (Fig. 1A and fig. S1). These spike proteins were either produced in human embryonic kidney (HEK) 293 mammalian cells (S1 and RBD) or insect cells (S1+S2 ectodomain and S2 domain). The purified S1+S2 ectodomain, the S1 domain, and the RBD proteins retained receptor binding activity as demonstrated by SPR assay using human angiotensin-converting enzyme 2 (hACE2) protein, the SARS-CoV-2 receptor (Fig. 1B). The S1+S2 ectodomain, S1 domain, and RBD demonstrated high-affinity interaction with hACE2 with affinity constants ranging from 5.09 to 37.23 nM. The control S2 domain protein (purple curve), lacking the RBD, did not bind to hACE2, demonstrating specificity of this receptor binding assay (Fig. 1B).

Female New Zealand white rabbits, a model frequently used to assess pharmacology/toxicology of vaccine antigens, were immunized twice intramuscularly at a 14-day interval with 50 μg of the purified proteins (two animals each) mixed with Emulsigen adjuvant. Sera
were collected before (pre-vaccination) and 8 days after the first and second vaccination and analyzed for binding antibodies in ELISA and SPR, pseudovirion and wild-type SARS-CoV-2 virus neutralization assay, and RBD competition assay and by GFPDL analysis.

**Antibody response after immunization with different spike antigens**

Serial dilutions of post-second vaccination rabbit sera were evaluated for binding of serum immunoglobulin G (IgG) to various spike proteins and domains in ELISA (S1+S2, black; S1, blue; RBD, red; and S2, purple) (fig. S2). Representative titration curves to spike ectodomain (S1+S2) and to the RBD in IgG-ELISA are shown in fig. S2 (A and B). End-point titers of the serum IgG were determined as the reciprocal of the highest dilution providing an optical density twice that of the negative control with no serum (fig. S2C). All four immunogens elicited strong IgG binding to the spike ectodomain (S1+S2). Binding to the individual domains (S1, S2, and RBD) was specific in that sera generated by S2 vaccination bound to S2 but not to S1 or RBD and vice versa.

SPR allows antibody binding to captured antigens in real-time kinetics, including total antibody binding in resonance units (MaxRU) in this figure for 10-fold serum dilution. All SPR experiments were performed twice, and the researchers performing the assay were blinded to sample identity. The variations for duplicate runs of SPR were <5%. The data shown are average values of two experimental runs. (D) Antibody off-rate constants were directly determined from the serum sample interaction with SARS-CoV-2 spike ectodomain (S1+S2), S1, S2, and RBD using SPR in the dissociation phase only for the sensograms with Max RU in the range of 20 to 100 RU. (E) RBD-hACE2 competition assay. Percent inhibition of hACE2 binding to RBD in the presence of 1:50 dilutions of post-second vaccination rabbit serum was measured by SPR. (F) End-point virus neutralization titers for one rabbit from each group using wild-type (wt) SARS-CoV-2 virus in a classical Biosafety Level 3 neutralization assay based on CPE (cytopathic effect) was performed as described in Materials and Methods. (G) Anti-spike ectodomain (S1+S2) binding antibody affinity as measured by antibody dissociation rates (off rates) of post-vaccinated rabbit polyclonal antibodies correlated with the wild-type (wt) SARS-CoV-2 virus end-point neutralization titers ($r = -0.9975; \text{P} < 0.005$). Pearson two-tailed correlations are reported for the calculation of correlations between anti-S1+S2 antibody affinity and end-point titers for one rabbit per immunogen. The color scheme in (G) is the same as in (D) or (F).
of RBD-hACE2 interaction, indicating the immunofocusing of S1+S2 post-vaccination sera demonstrated lower inhibition (mean, 44%) gave strong inhibition (mean, 94 and 84%, respectively), whereas the (at 1:50 serum dilution) from the RBD- and S1-vaccinated animals (fig. S5). In the RBD-hACE2 competition assay, the immune sera SPR assay (Fig. 1E) and in virus neutralization assays (Fig. 1F and fig. S5). In the RBD-hACE2 competition assay, the immune sera (at 1:50 serum dilution) from the RBD- and S1-vaccinated animals gave strong inhibition (mean, 94 and 84%, respectively), whereas the S1+S2 post-vaccination sera demonstrated lower inhibition (mean, 44%) of RBD-hACE2 interaction, indicating the immunofocusing of antibodies by RBD vaccination that blocks RBD-hACE2 receptor interaction.

SARS-CoV-2 neutralization was measured using SARS-CoV-2-FBLuc in a single-cycle pseudovirus neutralization assay in Vero E6 cells. The average percent inhibitions by post-first and post-second rabbit vaccination are shown in fig. S5A. Pre-vaccination rabbit sera (control Rb) did not neutralize SARS-CoV-2 in this assay. Sera generated by S1+S2 ectodomain, S1, and RBD (1:40 dilution) (but not anti-S2) showed 50 to 60% virus neutralization after a single vaccination and 93 to 98% virus inhibition by the post-second vaccination sera (fig. S5A). In addition, we determined the 50% end-point pseudovirus neutralization titers for the post-second vaccination rabbit sera. The RBD-immunized rabbits demonstrated two-fold higher IC50 (median inhibitory concentration) titers compared with spike ectodomain (S1+S2) and about threefold higher than S1 domain immune sera (fig. S5B). The end-point neutralization titers (PsVNS50) strongly correlated with serum antibody affinity against the spike ectodomain (S1+S2) (r = -0.9012; P = 0.0022) (fig. S5C).

Subsequently, we also conducted classical wild-type SARS-CoV-2 virus neutralization assay with 100 TCID50 (median tissue culture infectious dose) of SARS-CoV-2 (strain USA-WA1/2020 isolate). Although the end-point titers of this assay were lower than the IC50 titers observed in the pseudovirus neutralization assay (Fig. 1F versus fig. S5B), the RBD-immune serum demonstrated higher neutralization end-point titer (>1,640) compared with the S1 or S1+S2 immune sera (1:160 and 1:40, respectively). As shown with the pseudovirus neutralization titers, a strong inverse correlation was observed between the virus neutralization end-point titers and the post-vaccination serum antibody affinity (r = -0.9975; P = 0.0025) (Fig. 1G). Therefore, in three different functional assays, the RBD-immune sera showed better functional activity that correlated with the higher affinity of the antibodies in the post-second vaccination rabbit sera.

Epitope repertoires recognized by antibodies generated against SARS-CoV-2 spike antigens

The constructed SARS-CoV-2 GFPDL contains sequences ranging from 50 to 1500 base pairs (bp) long from the spike gene (GenBank no. MN908947) with >107.2 unique phage clones. The SARS-CoV-2 GFPDL displayed linear and conformational epitopes with random distribution of size and sequence of inserts that spanned the entire spike gene. SARS-CoV-2 GFPDL panning with individual post-second vaccination rabbit sera was conducted as described in Materials and Methods. The numbers of IgG-bound SARS-CoV-2 GFPDL phage clones with different serum samples ranged between 2.6 × 107 and 9.8 × 106 per milliliter (Fig. 2A). Graphical distribution of representative clones with a frequency of ≥2, obtained after affinity selection, and their alignment to the spike protein of SARS-CoV-2 are shown for the four vaccine groups (Fig. 2, B to E). The spike (S1+S2) ectodomain induced diverse antibody response that included strong binding to epitopes in the C-terminal region of the soluble protein spanning the HR2 region (i.e., multiple phage clones with similar inserts). This region may not be highly exposed on native virions or infected cells but is immunogenic in the soluble recombinant spike ectodomain. In addition, the rabbit anti-S1+S2 antibodies bound diverse epitopes spanning the RBD and, to a lesser degree, to the N-terminal domain (NTD), to the C-terminal region of S1, and to the N terminus of S2, including the fusion peptide (Fig. 2B and table S1). The S1 domain elicited very strong response against the C-terminal region of S1 protein and a diverse antibody repertoire recognizing
the NTD and RBD regions (Fig. 2C and table S1). The recombinant RBD induced high-titer antibodies that bound the largest number of phages \(9.88 \times 10^5\) (Fig. 2A) and were highly focused to the RBD/receptor binding motif (Fig. 2E and table S1). Binding to long epitope sequences (most likely conformational epitopes) was observed predominantly with the anti-RBD sera but much less for the anti-S1+S2 or anti-S1 immune rabbit sera. In contrast, the recombinant S2 immunogen after two immunizations in rabbits elicited antibodies primarily targeting the C terminus of the S2 protein (CD-HR2).

All the immunodominant antigenic sites identified by the SARS-CoV-2 GFPDL panning of all four immune sera on the spike sequence are shown in Fig. 2F and fig. S6. Alignment of the sequence with other coronaviruses shows that some of the antigenic sites are >70% conserved among several coronavirus strains isolated from humans and bats, especially sites located in the S2 domain (table S1). Structural depiction of these antigenic sites on the SARS-CoV-2 spike [fig. S7; in blue on Protein Data Bank (PDB) no. 6VSB] demonstrated that several of these antigenic sites identified here (primarily in and around RBD) are surface exposed on the native prefusion spike (3).

**DISCUSSION**

It is broadly accepted that an effective vaccine, which can be mass produced and deployed globally, will be required to curtail the current SARS-CoV-2 pandemic (6). Therefore, a better understanding of the humoral immune response generated by different vaccine antigens, including antibody epitope repertoire, antibody binding affinity, and functional activity, could greatly benefit the development and evaluation of vaccines against COVID-19.

In this study, we performed an in-depth evaluation of the antibody response generated by various SARS-CoV-2 spike antigens that are similar to the vaccine antigens being used in clinical development (6, 17, 18). A bioinformatics approach previously identified 279 potential B cell epitopes and 48 potential T cell epitopes in the spike glycoproteins of SARS-CoV, based on human antibody responses to...
SARS-CoV-1 infection and the corresponding epitopes in SARS-CoV-2 spike (19). Several of the predicted B cell epitopes overlapped with the sequences that we identified in our GFPDL analysis: amino acids 287 to 317 in NTD-RBD overlap with our antigenic site amino acids 298 to 363, which is 77% identical between SARS-CoV-1 and SARS-CoV-2; amino acids 524 to 598 and amino acids 601 to 640, in the C terminus of S1 overlap with our antigenic site containing amino acids 548 to 632 (78.8% conservation between SARS-CoV-1 and SARS-CoV-2); and amino acids 802 to 819 in the S2 domain/fusion peptide overlap with our antigenic site amino acids 768 to 828 (83% conserved between SARS-CoV-1 and SARS-CoV-2). Ten of 17 antigenic sites uniquely identified by the post-vaccination rabbit serum antibodies in the current study were probably missed by the prediction algorithms. This could be influenced by differences between antibody responses in rabbits and humans or may be due to sequence differences between SARS-CoV-1 and SARS-CoV-2, underscoring the limitations of the predictive algorithms in the in silico approach. However, confirmation of exposure of these antigenic sites on the trimeric form of the SARS-CoV-2 spike and on SARS-CoV-2 virions will require further investigation. One of the possible limitations of GFPDL-based assessments is that although the phage display is likely to detect both conformational and linear epitopes on the CoV-2 spike proteins, they are unlikely to detect paratopic interactions that may require post-translational modifications or quaternary epitopes formed by cross-protomers.

Unexpectedly, the S2 domain does not appear to elicit as many neutralizing antibodies as RBD or S1. Although S2 contains the fusion peptide, it does not appear to be as immunogenic, compared with S1 or RBD, in generating binding antibodies to the intact spike (S1+S2) ectodomain, as observed in both IgG ELISA and SPR. It is possible that in addition to the RBD, additional antigenic sites, for example, amino acids residues 548 to 632 located close to the RBD in the S1 domain C terminus, may also contribute to the neutralizing potential of antibodies.

Although we characterized the purified proteins in various assays, there is a possibility that the structure of the antigens used in the study is different from the corresponding authentic spike protein on the surface of SARS-CoV-2 virion particle. These commercial antigens were also manufactured in different systems (insect or mammalian cells), so differential glycosylation and other modifications may be present. This study used two rabbits per immunogen and was designed to be qualitative rather than quantitative. It will be important to investigate how the vaccination-induced immune responses in rabbits compare with immune responses in humans who are either vaccinated with candidate vaccines or COVID-19 survivors. Moreover, the rabbits were not challenged with virus, so we cannot determine whether the antibodies induced by vaccination were protective.

One unexpected finding in this study was the higher affinity of antibodies elicited by the RBD compared with the other spike antigens (S1+S2 ectodomain, S1, and S2 domains), which strongly correlated with the neutralization titers. In studies related to other viral infections, high-affinity antibody responses were associated with clinical benefit in model systems or infected individuals (13, 14, 19, 20). Thus, vaccines that can elicit high-affinity antibodies may provide a considerable advantage for clinical outcome of SARS-CoV-2 infection and contribute to amelioration of disease in infected individuals. Therefore, in addition to measurements of antibody binding titers and virus neutralization, this and the previous studies demonstrate the importance of assessments of antibody affinity maturation during SARS-CoV-2 vaccine trials. In addition to designing the best immunogen, selection of appropriate adjuvant could drive responding B cells into affinity maturation in germinal centers, as previously observed in clinical trials of influenza vaccines (10, 11).

In summary, our study highlights the need to perform comprehensive analysis of immune response generated after vaccination or SARS-CoV-2 infection to identify biomarkers of protective immunity. In-depth understanding of quantitative and qualitative aspects of immune responses generated by different spike protein vaccine antigens could aid the development and evaluation of effective SARS-CoV-2 therapeutics and vaccines.

**MATERIALS AND METHODS**

**Study design**

The objective of this study was to investigate humoral immune response after vaccination with different SARS-CoV-2 spike immunogens. We used purified recombinant spike antigens to immunize female New Zealand white rabbits. Because of time, funding, and staffing restrictions during the COVID-19 pandemic, we began the exploratory study with eight rabbits and maximized the number in each group (n = 2). Using analysis of variance (ANOVA) to compare between the two groups with a sample size of 2, we needed a power of 0.0952 to achieve an effect size of 0.5 with a significance level of 0.05. Samples and assays were run in duplicate or triplicate when possible after consideration of ethical animal sampling guidelines. Research fellows running the antibody assays were blinded to the identity of the groups for assessments of outcomes. Primary data are reported in data file S1.

**Recombinant CoV proteins**

Recombinant SARS-CoV-2 proteins were purchased from Sino Biologicals (S1+S2 ectodomain, 40589-V08B1; S1, 40591-V08H; RBD, 40592-V08H; or S2, 40590-V08B). Recombinant purified proteins used in the study were either produced in HEK 293 mammalian cells (S1 and RBD) or insect cells (S1+S2 ectodomain and S2 domain).

**Rabbit immunization studies**

Female New Zealand white rabbits (Charles River Laboratories) were intramuscularly immunized twice at 14-day intervals with 50 µg of purified recombinant proteins mixed with Emulsigen adjuvant (MVP Adjuvants). Two animals were used per immunogen. All animal experiments were approved by the U.S. Food and Drug Administration (FDA) Institutional Animal Care and Use Committee under protocol no. 2008-10. The animal care and use protocol meets National Institutes of Health guidelines. Sera were collected before (pre-vaccination) and 8 days after the first and second vaccination and analyzed for binding antibodies in ELISA, SPR, and neutralization assay and GFPDL analysis.

**ELISA**

Immulon plates (96 wells) were coated with recombinant spike immunogens (100 ng/100 µl; protein and protein domains) in phosphate-buffered saline (PBS) overnight at 4°C. Starting at a 1:100 dilution, serum samples were serially diluted 1:5 and applied to the protein-coated plate in 10 µl for 1 hour at ambient temperature. Serum samples were assayed in duplicate. Naïve serum samples were assayed along with the experimental samples. After three
was measured using luciferin-containing substrate. Controls included cell-only control, virus without any antibody control, and positive control sera.

**Classical wild-type SARS-CoV-2 virus neutralization assay**

One hundred TCID$_{50}$ of SARS-CoV-2 (isolate USA-WA1/2020) was incubated with two-fold serial rabbit serum dilutions in a round-bottom plate at 37°C for 1 hour. The virus-antibody mixture was then added to a 96-well plate with 5 × 10$^4$ Vero E6 cells. After 1 hour, the mixture was removed and replenished with fresh minimum essential medium containing 2% fetal bovine serum. Cells were incubated at 37°C for an additional 72 hours; then, cytopathic effect was measured by CellTiter-Glo luminescence assay (Promega). Luciferase abundance was determined using Veritas luminometer. The end-point titers were calculated as the last serum dilution resulting in at least 50% SARS-CoV-2 neutralization.

**SPR-based RBD-hACE2 binding and inhibition assay**

The recombinant SARS-CoV-2 RBD protein from HEK 293 cells (RBD) was captured on a sensor chip with 500 RU in the test flow channels. Samples of 200 μl of freshly prepared post-second immunization rabbit sera at a 10-fold dilution were injected at a flow rate of 50 μl/min (contact duration, 180 s) for association. After antibody binding, recombinant hACE2 (1 μg/ml; ACROBiosystems) was injected at a flow rate of 50 μl/min (contact duration, 120 s) for association. Responses from the protein surface were corrected for the response from a mock surface and for responses from a buffer-only injection. SPR was performed with serially diluted serum of each animal in this study.

**GFPDL construction**

The SARS-CoV-2 spike gene (GenBank no. MN908947) was chemically synthesized (GenScript) and used for cloning and construction of phage display libraries. A gIII display–based phage vector, fSK-9-3, was used where the desired polypeptide can be displayed on the surface of the phage as a gIII-fusion protein. Purified DNA containing the spike gene was digested with deoxyribonuclease I to obtain gene fragments of 100- to 1000-bp size range and used for GFPDL construction as described previously (7–9). The phage libraries constructed from the SARS-CoV-2 spike gene display viral protein segments, ranging in sizes from 30 to 350 amino acids as fusion proteins on the surface of bacteriophage.

**Affinity selection of SARS-CoV-2 GFPDL phages with polyclonal rabbit serum**

Before panning of GFPDL with polyclonal serum antibodies, serum components that could nonspecifically interact with phage proteins were removed by incubation with ultraviolet-killed M13K07 phage-coated petri dishes (9). Equal volumes of each post-vaccination rabbit serum were used for GFPDL panning. GFPDL affinity selection was carried out in-solution with protein A/G (IgG)–specific affinity resin as previously described (7, 8, 10). Briefly, individual rabbit serum was incubated with the GFPDL and the protein A/G resin, and the unbound phages were removed by PBST (PBS containing 0.1% Tween-20) wash followed by washes with PBS. Bound phages were eluted by addition of 0.1 M Gly-HCl (pH 2.2) and neutralized by adding 8 μl of 2 M tris solution per 100 μl of eluate. After panning,
antibody-bound phage clones were amplified, the inserts were sequenced, and the sequences were aligned to the SARS-CoV-2 spike gene to define the fine epitope specificity in the post-vaccination rabbit sera. The GFPDL affinity selection was performed in a blinded fashion. Similar numbers of bound phage clones and epitope repertoire were observed in the two GFPDL panning experiments.

**Sequence and structural alignments**

Spike protein sequences of SARS-CoV-2 (GenBank no. MN908947), SARS-CoV-1 BJ01 strain (GenBank no. AAP30030.1), MERS CoV KOR/KNIH/2015 (GenBank no. AKN10175.1), bat SARS-like CoV ZC45 (GenBank no. AVP78031.1), bat SARS-like CoV ZXC21 (GenBank no. AVP87042.1), bat CoV BM48-31/BGR/2008 (GenBank no. ADK66841.1), human CoV 2c EMC/2012 (GenBank no. AF88936.1), human CoV NL63 [National Center for Biotechnology Information (NCBI) no. YP_003767.1], and human CoV HKU1 (NCBI no. YP_173238.1) were used for alignment. Structural alignments were depicted on SARS-CoV-2 spike prefusion structure (PDB no. 6VSB) (3).

**Statistical analysis**

Correlations were calculated with the Pearson method, and a P value for correlation was calculated by two-tailed test.

**SUPPLEMENTARY MATERIALS**

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Fig. S1. Purified SARS-CoV-2 proteins analyzed by SDS–polyacrylamide gel electrophoresis for correlation was calculated by two-tailed test.

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Spike selection
A vaccine for SARS-CoV-2 is urgently needed. Ravichandran et al. immunized rabbits with different SARS-CoV-2 spike proteins to profile the quality of induced antibody responses. Although all antigens produced neutralizing antibodies, immunization with the receptor binding domain (RBD) led to the highest affinity antibodies. The authors went on to map epitopes on the spike protein recognized by the rabbit antibodies. This qualitative study could inform antigen selection for SARS-CoV-2 vaccines.
