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TITLE:
Roadmap to developing a recombinant coronavirus S protein receptor-binding domain vaccine for severe acute respiratory syndrome

ABSTRACT:
A subunit vaccine, RBD-S, is under development to prevent severe acute respiratory syndrome (SARS) caused by SARS coronavirus (SARS-CoV), which is classified by the US NIH as a category C pathogen. This vaccine is comprised of a recombinant receptor-binding domain (RBD) of the SARS-CoV spike (S) protein and formulated on alum, together with a synthetic glucopyranosyl lipid A. The vaccine would induce neutralizing antibodies without causing Th2-type immunopathology. Vaccine development is being led by the nonprofit product development partnership; Sabin Vaccine Institute and Texas Children's Hospital Center for Vaccine Development in collaboration with two academic partners (the New York Blood Center and University of Texas Medical Branch); an industrial partner (Immune Design Corporation); and Walter Reed Army Institute of Research. A roadmap for the product development of the RBD-S SARS vaccine is outlined with a goal to manufacture the vaccine for clinical testing within the next 5 years.

Antigen ::: Proposed product:
The recombinant protein is comprised of a 193 amino acid (21 kDa) polypeptide corresponding to the minimal RBD (amino acid residues: 318–510) of the SARS-CoV S protein [4]. The full-length SARS-CoV S protein belongs to the class I of viral fusion proteins, which also includes the glycoprotein gp160 of HIV and hemagglutinin of influenza virus [5,6]. The S protein fragment spanning the 193-mer polypeptide is the minimal RBD responsible for virus binding to the host-cell receptor, angiotensin-converting enzyme 2 [4,7].
RBD-S, which contains multiple conformation-dependent epitopes, induces strong neutralizing antibody responses, CD8+ T-cell responses and long-term protective immunity in animal models, as well as potent cross-neutralizing antibodies against pseudoviruses expressing S proteins of 2002–2003 and 2003–2004 human SARS-CoV strains, and an isolate from a civet zoonotic host [8–11]. Moreover, more than 50% of the neutralizing activity in the sera of convalescent SARS patients is mediated by RBD-S-specific antibodies [9,12], while RBD-S can absorb and remove the majority of neutralizing antibodies in the antisera of rabbits, mice and monkeys immunized with inactivated SARS-CoV or modified vaccinia virus expressing S protein [9,13]. These studies provide the rationale for selecting RBD-S as an important target for vaccine development. The recombinant 21 kDa RBD-S will be expressed in yeast (Pichia pastoris), bacteria (Escherichia coli), or baculovirus (pending studies demonstrating optimal protection, as described below). More recently, a second RBD-S 219-mer construct corresponding to residues 318–536 was expressed in Chinese hamster ovary-K1 cells and shown to exhibit correct folding and conformation followed by equivalent or enhanced protection [14]; this construct will also be evaluated as a back-up vaccination strategy.

Adjuvant ::: Proposed product:
GLA is a 1.7 kDa synthetic TLR4 agonist functionally equivalent to monophosphoryl lipid A, a nontoxic derivative of the lipopolysaccharide [15–18]. In a study in mice with a commercial killed influenza vaccine, chemically synthesized GLA was effective in enhancing IgG1 and IgG2a titers, both as an oil–water emulsion (squalene oil and surfactant) and as an aqueous formulation [15,16]. Subsequent studies revealed that GLA also enhances T-cell responses to influenza vaccine among older adults [18], which would be a desirable feature for a SARS vaccine.

Indication & target product profile of the RBD-S SARS vaccine:
The vaccine is initially indicated for adults and individuals over the age of 15 who are considered at greatest risk of mortality from SARS [2]. The vaccine would be administered as an injectable product to protect against lethal SARS-CoV infection, either as a single dose or two doses spaced closely together (within 2–4 weeks), so that vulnerable populations could be rapidly immunized in an outbreak setting (Table 1). The proposed target product profile also accounts for the stability of the RBD-S SARS vaccine (up to 4 years at 2–8°C) to allow for emergency stockpiling and to protect at low-cost at-risk populations in underdeveloped and developing

countries (particularly southeast Asia where the risk of reemergence is greatest), as well as developed countries.

Comparative advantage of an RBD-S SARS vaccine:
Several different technologies have been proposed for developing human SARS-CoV vaccines, including inactivated virus vaccines, live–attenuated vaccines, adenovirus or poxvirus vectored vaccines, and DNA vaccines [1]. Among the concerns regarding these vaccines is the possibility of vaccine enhancement of disease, which has been noted with feline CoVs causing feline infectious peritonitis [1,19,20]. Similarly, laboratory animals administered alum-adjuvanted whole-virus SARS-CoV vaccine and subsequently challenged with infectious virus experienced enhanced disease and immunopathology comprised of eosiniophilic infiltration and Th2-mediated inflammatory alveolar damage [21], similar to immunopathologic lung reactions found in infants and animals challenged with respiratory syncytial virus (RSV) [22,23]. A double-inactivated SARS-CoV (formalin and ultraviolet radiation) was also shown to elicit eosinophilic and immunoenhancing pathology, as well as poor protection, especially in aged animals, suggesting the possibility that aged humans (a desired target population for the vaccine) may be particularly vulnerable to vaccine-induced effects [24]. Further studies showed that a Venezuelan equine encephalitis vector containing the SARS nucleocapsid (NP) gene also elicited eosinophilic immunopathology in BALB/c mice after challenge without any noticeable protection [25]. Thus, it was hypothesized that the NP antigen of SARS-CoV may be responsible for this property of inactivated vaccines [25,26]. It was also reported that the formalin-inactivated RSV vaccine was associated with lack of antibody affinity maturation followed by poor TLR stimulation, suggesting that NP may not account entirely for immune enhancement [27].
Both Venezuelan equine encephalitis vector containing S-expressing plasmid and alum-adjuvanted recombinant protein (expressed in baculovirus) were shown to elicit protection in BALB/c mice when challenged with live SARS-CoV [28]. However, it remains unclear whether such S protein constructs also elicit eosinophilic pathology. One report, for example, has indicated that S protein administered either as a baculovirus-expressed and purified recombinant protein or as a virus-like particle, causes Th2-type pathology with eosinophils, albeit reduced when compared with whole virus or NP protein constructs [28]. Similarly, a SARS vaccine candidate based on recombinant, properly folded, native full-length S protein trimers (expressed in mammalian cells) was also shown to elicit some immune enhancement [29].
Thus, while the recombinant S protein elicits protection, its use as a human vaccine might be limited by host immunopathology.
In order to re-engineer the S protein as a safe and effective vaccine, early studies indicate that the RBD component is highly protective in laboratory animals, while significantly reducing the risk of antibody enhancement of disease [5,6,8–12]. Additional data confirm that sera from SARS convalescent patients contain robust neutralizing antibodies to RBD-S [9,12].
To summarize, our vaccine technology has the potential advantages over existing vaccine technologies that have previously been reported:
• The RBD-S subunit vaccine is expected to be safer than other SARS vaccines described above because the latter may elicit eosinophilic immunopathology or antibody-mediated enhancement of disease, while our vaccine is not expected to cause harmful immune responses.• The RBD-S subunit vaccine may induce more potent cross-neutralizing antibody responses than other SARS vaccines reported.

Scientific & technical value of the project:
The goal and deliverable is to produce a formulated recombinant RBD-S vaccine under current good manufacturing practices (cGMP), in preparation for clinical testing to assess safety, immunogenicity and efficacy against human SARS-CoV infection.
The key technological objectives involve developing a process for vaccine manufacture utilizing a bacterial, yeast or baculovirus expression system, as well as an inexpensive purification method that maximizes the production of RBD-S antigen component of the vaccine at a minimum cost. These activities would take place through a consortium led by Sabin Vaccine Institute and Texas Children's Hospital Center for Vaccine Development ('Sabin-TCH'), an established PDP with more than a decade-long track record of transitioning recombinant protein vaccines through process development, cGMP and clinical testing [30]. PDPs are nonprofit organizations that use industry practices and often partner with for-profit organizations, including biotechnology and multinational pharmaceutical companies, as well as academic and other partners [30]. To develop the RBD-S SARS vaccine, Sabin-TCH will work with the Immune Design Corporation (Seattle, WA, USA) for

adjuvant access and formulation, and with the Lindsley F Kimball Research Institute of the New York Blood Center (New York, NY, USA) and the University of Texas Medical Branch (Galveston, TX, USA), in the areas of assay development and confirmatory preclinical efficacy testing.
The Walter Reed Army Institute of Research (WRAIR) will take the lead on the final cGMP manufacture (Figure 1). Batch production records will be utilized to ensure reproducibility and documentation under a quality umbrella. Before transferring technology to the WRAIR Pilot Bioproduction Facility for cGMP manufacture, the final process will be repeated a minimum of three times at the 10-l scale to assure that the vaccine manufacture, from fermentation through formulation, reproducibly results in a product of sufficient yield and quality. In addition, antigen/adjuvant formulation studies will be established both biochemically and by immunological assessment (potency). By performing a minimum of three full pilot runs with quality control for both process and release, the authors will maximize the probability of successful technology transfer for the cGMP manufacture and with optimal yield and purity. Accelerated and long-term stability studies will be conducted on the final formulations, with chemical stability emphasizing provocative tests at high pH to induce deamidation and with selective oxidizing agents to produce oxidation. In addition, stability will be assessed at different time points after conducting temperature incursion experiments (repeated freeze–thaw cycles and storage at different temperatures). Following completion of process development of the vaccine, the production technology will be transferred to WRAIR for cGMP manufacture. The final product will be subjected to a rigorous battery of assays for lot and product release at Sabin-TCH, WRAIR and other contractors (Table 2). Following GLP toxicology testing, a regulatory filing for clinical testing will be submitted to the US FDA.
The major innovations for this product include:
• Producing the RBD-S polypeptide formulation, rather than the complete S protein, with the use of GLA, a novel TLR4 agonist, at point of injection in order to limit vaccine-induced Th2-type immunopathology enhancement.• The long-term stability of the vaccine will be maximized by evaluating the biochemical and biophysical characteristics [31,32]. Such activities are essential for a vaccine that might require emergency stockpiling. Biophysical assessment is initiated evaluating the recombinant protein using multiple biophysical techniques, including circular dichroism, intrinsic and extrinsic fluorescence, light scattering, and differential scanning calorimetry [31,32]. Collectively, these studies provide specific information concerning the physical state of the protein as a function of temperature and pH. Biophysical data are then used to design high-throughput screening assays for the identification of potential stabilizers, typically GRAS (generally regarded as safe)-designated excipients and stabilizers that would be acceptable to the FDA and other regulatory agencies [32].• Preclinical testing in an innovative human angiotensin-converting enzyme 2 transgenic (Tg) mouse model [33,34]. Following intranasal infection of such Tg mice with SARS-CoV, weight loss and other clinical manifestations become apparent before reaching 100% mortality within 8 days post-infection. In contrast, infected transgene-negative (Tg) mice survive without showing any clinical illness [33]. The severity of the disease developed in these Tg mice makes them valuable for preclinical testing of SARS-CoV vaccines [33,34].

Summary of immediate project goals & key technological objectives:
The first major deliverable will be an RBD-S protein-based SARS vaccine approved for Phase I clinical testing by the FDA.

Product & clinical development challenges:
The major product challenges includes: selection of antigen expression systems and demonstrating feasibility of scale-up expression with process development at acceptable yields, purity, and stability; preclinical optimization for testing immunological responses and efficacy in laboratory animals; technology transfer for cGMP manufacture; completion of a GLP toxicology study and a clinical development plan that meets requirements of the FDA for ‘the animal rule’; and immune correlates of protection [102].

Selection of antigen expression systems & feasibility of scale-up expression:
Three protein expression systems, bacterial (E. coli), yeast (P. pastoris) and baculovirus (and possibly mammalian cells), will be evaluated in parallel for the feasibility of scalable expression of the recombinant RBD-S protein. Preliminary studies have shown that E. coli-expressed RBD-S protein was able to elicit high levels of neutralizing antibodies and protection in an animal model [6]. However, the E. coli-expressed protein was not evaluated and formally characterized for its scalability; hence, a more formal evaluation is proposed.

Alternatively, Sabin-TCH has demonstrated that P. pastoris is a very robust and sometimes simpler, system for expression. Through Sabin's Human Hookworm Vaccine Initiative, at least two hookworm vaccine antigens, Na-ASP-2 and Na-GST-1, have already been shown to be suitable for scalability, producing high resolution crystals for X-ray diffraction [35–38], as well as antigens suitable for clinical development [39,40]. For RBD-S, the optimal expression systems will be selected for the subsequent studies based on the following criteria: yield, scalability, purity and stability profile of the RBD-S protein expressed; recognition of the RBD-S by conformational monoclonal antibodies and by human convalescent sera; ability of the RBD-S to inhibit viral infection in vitro; and ability of the RBD-S to induce neutralizing antibodies and other protective immune responses in vaccinated mice against subsequent lethal challenge with homologous and heterogeneous strains of SARS-CoV.

Preclinical optimization & immunological testing:
Using the RBD-S protein from the selected expression system and adjuvant formulation, the authors will optimize the immunization regimens to induce the highest levels of immune responses, particularly neutralizing antibodies and protection against subsequent challenge. Immunological and protection studies using wild-type and Tg mice will require optimization in terms of antigen dose and formulation; number and interval of immunizations and route of administration; evaluation of efficacy of the optimized regimen; extended studies to optimize long-term immunological responses and length of protection; and no vaccine-induced Th2-derived immunopathology.

Process development, characterization, formulation & stability evaluation:
Following selection of an expression system, a research cell bank will be generated, and expression of RBD-S in a 10-l bioreactor (fermentation) will be optimized. To improve yield, purity, stability and activity of the soluble protein, the optimal growth and induction conditions for the fermentation processes will be determined first using flasks. Once the best parameters are selected, they will be evaluated at the 10-l scale, with the aim of obtaining a reproducible high-yield production (minimum of 100 mg/l target). The major components of this activity will include: production of the RBD-S protein at the 10-l fermentation scale and purification using ion-exchange, hydrophobic interaction or other column chromatographies; documentation of three successive process development runs (fermentation and protein purification); development of product-specific assays to monitor the antigen identity, purity and integrity during fermentation and purification, and for clinical lot release; development of buffer and adjuvant formulations, and product stability studies.

Technology transfer, cGMP manufacture & lot release, GLP toxicology & IND preparation:
The process development and formulation technology for the selected RBD-S vaccine will be transferred to WRAIR. Developed and qualified assays will be used for lot release and stability/potency testing. The protein-specific assay will be conducted at Sabin-TCH (Table 2), while other regulated assays will be contracted to industrial partners.
These assays can provide important feedback about the purity, stability and integrity of the molecule essential to guide process development and formulation. Additionally, new assays and technology are typically identified and implemented, where appropriate, to ensure specific characteristics of the molecule are maintained. Several of these assays, either currently used or being developed for RBD-S protein, have been qualified by Sabin-TCH for other recombinant antigens for Phase I clinical trials. Following a pre-IND meeting with the FDA, a GLP toxicology testing will be performed. Sabin Vaccine Institute will serve as the sponsor and prepare the IND for the vaccine. The initial design of a global access roadmap for vaccine uptake and distribution will also be drafted.

Clinical development & pivotal animal efficacy studies:
The safety of RBD-S vaccine will be evaluated in a Phase I clinical trial. Under a new subpart of the US Code of Federal Regulations, it is acceptable to approve new drugs through pivotal animal efficacy studies based on the 'Animal Rule' [41] when human efficacy studies are not ethical or feasible because they would deliberately expose healthy human volunteers to a lethal agent [41]. While safety and immunogenicity studies with the RBD-S vaccine are feasible, human challenge studies with SARS-CoV would not be considered safe or ethical.
Therefore, pivotal animal efficacy studies will be conducted using at least one animal model exhibiting host pathogenic and immune responses that are matched as closely as possible to

those of humans [41]. Besides the nonhuman primate model, the diseases and lethality revealed by ACE2 transgenic mice infected by clinical isolates of SARS-CoV (e.g., Urbani) and those elicited by wild-type Balb/c mice in response to a mouse-adapted strain of SARS-CoV, designated MA-15) [33,34,42] make them among the best models for evaluating in vivo efficacy of SARS vaccines.
Determination of the virus neutralizing antibody titers among vaccine recipients in the pivotal animal efficacy study is an important bridging of animal protection data to humans. To serve as an immune correlate of protection, antibody titers would have to equal or exceed the neutralizing antibody titers and amounts found in the sera of convalescent patients, with desired quantities of specific neutralizing antibodies to be determined in consultation with expert clinical virologists. In addition, levels of antibody affinity and avidity may need to be established using surface plasmon resonance and other technologies [43].

Expert commentary:
Following the initial discovery of the SARS-CoV as the etiologic agent of human SARS in 2003, an international effort has been underway to develop and test prototype vaccines. These intensive studies determined that when delivered as an injectable vaccine, inactivated SARS-CoV can elicit protective neutralizing antibodies. However, such vaccines also caused a Th2-derived immunoenhancing pathology bearing resemblance to the immunopathology that derailed efforts to produce an inactivated RSV vaccine more than four decades ago. Subsequent efforts determined that protective neutralizing antibodies were directed primarily against S protein responsible for receptor-binding, but even vaccines comprised of the full-length S protein can elicit immunopathology, albeit in reduced amounts. Therefore, efforts have, instead, focused on a subunit vaccine comprised of only the 193-mer RBD-S, the essential component responsible for receptor binding. In laboratory animals, recombinant RBD-S subunit vaccines elicited protection comparable with the S protein-based vaccines, but with minimal immune enhancement of immunopathology. A prototype recombinant RBD-S SARS vaccine formulated on alum, together with GLA at point of injection, is under development by the activities of a nonprofit PDP in collaboration with key academic, industrial and military partners.

Five-year view:
Within 5 years, it is anticipated that a prototype recombinant RBD-S SARS vaccine formulated on alum, and with GLA at point of injection, will have completed cGMP manufacture at WRAIR. Following lot release and GLP toxicology testing, the vaccine will be ready for an IND submission and Phase I clinical testing. A full clinical development plan leading to product licensure will need outside consultation to confirm the quantity and quality, as well as affinity and avidity, of virus neutralizing antibodies required for protection.