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A recombinant baculovirus-expressed S glycoprotein vaccine elicits high titers of SARS-associated coronavirus (SARS-CoV) neutralizing antibodies in mice

Zhimin Zhou [a,*], Penny Post [a], Rick Chubet [a], Katherine Holtz [a], Clifton McPherson [a], Martin Petric [b], Manon Cox [a]

[a] *Protein Sciences Corporation, 1000 Research Parkway, Meriden, CT 06540, USA*
[b] *BC Centre for Disease Control, 655W 12th Ave, Vancouver, BC, Canada V5Z 4R4*

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Abstract

A recombinant SARS-CoV spike (S) glycoprotein vaccine produced in insect cells in a pre-clinical development stage is described. A truncated version of S glycoprotein, containing only the ecto-domain, as well as a His-tagged full-length version were cloned and expressed in a serum-free insect cell line, *ExpresSF*+®. The proteins, purified to apparent homogeneity by liquid column chromatography, were formulated without adjuvant at 3, 9, 27, and 50 μg per dose in phosphate saline and used to immunize mice. Both antigens in each formulation elicited a strong immune response after two or three vaccinations with the antigen. Neutralizing antibody titers correlated closely with standard ELISA reactivity against the S glycoprotein. The truncated S protein was also formulated with an adjuvant, aluminum hydroxide, at 1 μg per dose (±adjuvant), and 5 μg per dose (±adjuvant). Significantly enhanced immune responses, manifested by higher titers of serum ELISA and viral neutralizing antibodies, were achieved in adjuvanted groups with fewer doses and lower concentration of S glycoprotein. These findings indicate that the ecto-domain of SARS-CoV S glycoprotein vaccine, with or without adjuvant, is immunogenic and induces high titers of virus neutralizing antibodies to levels similar to those achieved with the full S glycoprotein vaccine.
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Keywords: SARS-CoV; Recombinant protein vaccine; Virus neutralization; Adjuvant; Aluminum hydroxide

1. Introduction

Severe acute respiratory syndrome (SARS) is a respiratory disease, whose main symptoms include fever, cough, shortness of breath, and pneumonia. The World Health Organization reported 8114 cases with 775 fatalities (WHO: Updated recommendations, October, 2004). The etiologic agent of SARS was shown to be a coronavirus, now designated as SARS-CoV [1–5]. The disease has the potential to re-appear as a new naturally acquired outbreak, by accidental- [6], or by intentional-release.

The development of SARS-CoV vaccines has become a priority for preparedness for future outbreaks to protect health care workers managing new cases as well as the general population. A Phase I clinical trial using killed-whole SARS-CoV vaccine has been conducted by Sinovac Biotech Ltd. However, concerns have been raised for the use of inactivated virus vaccines based on observations with feline coronavirus. There are also several subunit vaccines in pre-clinical development, targeting SARS-CoV structural proteins, including the spike (S) and nucleocapsid (N) proteins. Other vaccine candidates include vector-based DNA vaccines [7–11], a combination of whole killed virus and DNA vaccines [12], inactivated whole virus vaccines [13,14], a combination of DNA and S-peptide vaccines [15], and other recombinant proteins and their fragments.

* Corresponding author. Tel.: +1 203 686 0800; fax: +1 203 686 0268.
E-mail address: zzhou@proteinsciences.com (Z. Zhou).

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One of the most promising candidates for SARS-CoV protein vaccine is the S glycoprotein protein. Oligomers of this glycoprotein form large spikes in the viral envelope and mediate the binding of SARS-CoV to host cell through host cell receptor, the angiotensin converting enzyme II (ACE2) [16–19]. The receptor-binding domain (RBD) of the S glycoprotein has been mapped to residues 318–510 [20–22]. A complex structure of RBD and soluble ACE2 was determined at 2.9 Å [23]. The S protein and its fragments were shown to also induce neutralizing antibodies [16,24–33].

The S glycoprotein has been biochemically characterized [20,34], and its features were explored for therapeutic applications [20,35].

This report describes two pre-clinical studies in mice using purified subunit vaccines of SARS S glycoprotein consisting of the ecto-domain (transmembrane domain deleted) of this protein and a His-tagged full-length S glycoprotein, expressed in an insect cell system. Immunogenicity of the protein vaccines in an ELISA and in viral neutralizing antibody assays was determined together with the adjuvant effect of aluminum hydroxide.

2. Materials and methods

2.1. Recombinant protein vaccine

The gene encoding the S glycoprotein was cloned from a lysate of culture SARS-CoV 3200300841 (Passage #3) in Trizol LS Reagent (Sigma), which was kindly provided by Dr. Dean Erdman (CDC, Atlanta). Briefly, the cDNA was cloned in two steps due to the size of the gene (∼3.5 kb). The 5′ one-third of the ORF (Front), containing the N-terminus, was cloned into a baculovirus transfer vector pPSC12 (Protein Sciences Corporation, PSC), downstream from the baculovirus very late promoter of the polyhedrin gene. The insert was also in frame with the chitinase secretion signal sequence. The remainder of the gene was cloned into pUC18. Both inserts were sequenced (MWG Biotech) in full to confirm presence of entire gene. Two fragments of the ORF were then joined in pPSC12 using convenient restriction sites to assemble the complete SARS-CoV ORF and the insert and flanking regions of the vector were sequenced.

The regions encoding the transmembrane and cytoplasmic domains of SARS-CoV were deleted using site-directed PCR. This construct was named ΔTM S (Fig. 1A). To assist purification of the full-length S protein, a His_6-tag was constructed to the C-terminus of the protein (Fig. 1A). Both constructs were sequenced in full and their nucleotide sequences were identical to the published sequence of SARS-CoV S gene for the protein region (Gen Bank Accession No. AY278741).

To generate a recombinant baculovirus, linearized *Autographa California* nuclear polyhedrosis virus (AcNPV) DNA, and the recombination plasmid DNA containing the S gene were mixed, co-precipitated with calcium phosphate,

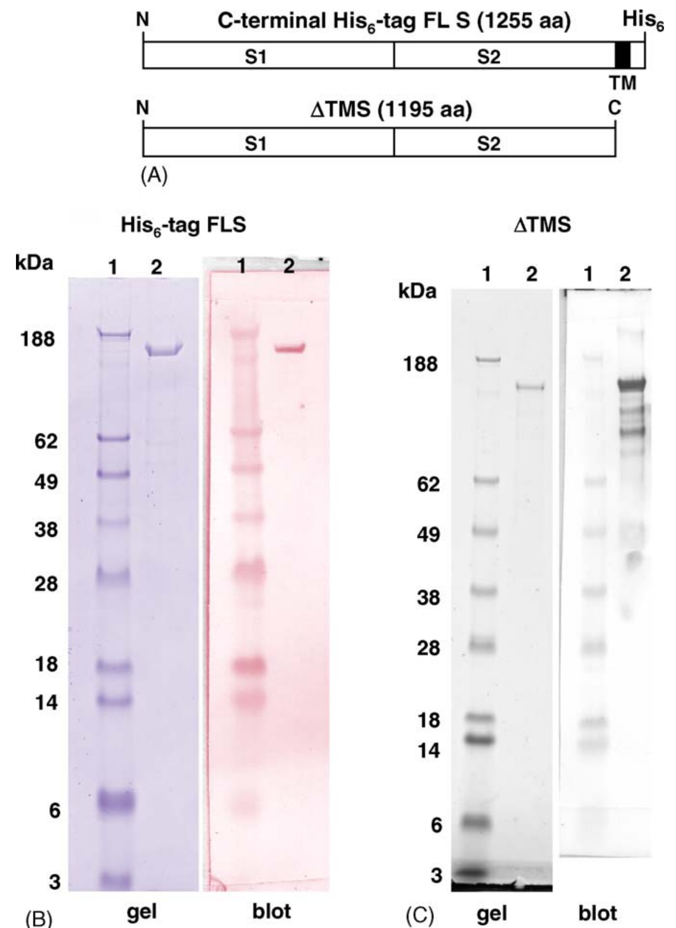

Fig. 1. Two recombinant SARS spike proteins used in the study, C-terminal His_6-tagged full-length S (His_6-tag FL S) and the ecto-domain of S (transmembrane domain deleted, ΔTM S) proteins. (A) schematic representations of the S proteins. (B) purified His_6-tag FL S. (C) purified ΔTM S.

and Sf9 cells were transfected. Recombinant viruses were identified by their distinctive plaque morphology. A single recombinant virus plaque was isolated. The virus was further amplified using Sf9 cells for P1 and *ExpresSF*+® cells in the absence of serum for P2 and P3. The recombinant virus was further scaled up before added to 45 liters of *ExpresSF*+® cell culture in a bioreactor at a multiplicity of infection of 1.0 plaque forming unit (pfu)/cell. The infected culture was incubated at 27 °C for 60–64 h. During this period, recombinant SARS-CoV ΔTM S protein was expressed in and secreted from the recombinant baculovirus-infected cells, while the His-tagged full-length S glycoprotein was not secreted.

Both ΔTM S and His-tagged full-length S glycoproteins were purified to apparent homogeneity by column chromatography (Fig. 1B and C) before formulation. Protein identity was determined by N-terminal peptide sequencing and Western blotting with anti-His_6-tag monoclonal antibody (QIAGEN), and/or anti-SARS S sera (PSC). Purity of His-tagged full-length S (>90%) and ΔTM S (>95%) was determined by densitometry (Quantity One Densitometry

Table 1
Mouse immunogenicity study without adjuvant

Group	Test article	Dose (μg)	# Mice/day of blood sampling					
			Day 1[a]	Day 15[a]	Day 30[a]	Day 45[a]	Day 60	Day 75
1	ΔTM S	3	–	8	8	8	8	8
2	ΔTM S	9	–	8	8	8	8	8
3	FL His-tag	9	–	8	8	8	8	8
4	ΔTM S	27	–	8	8	8	8	8
5	ΔTM S	50	–	8	8	8	8	8
6	Vehicle	–	8	–	4	–	4	4

[a] Dose day. Blood was collected prior to the administration of test article. For example, on Day 15, eight mice, half male and half female, of the total 40 mice in Group 1 that received one dose (50 μl) were bled. The rest of 32 mice were immunized with the second dose of 3 μg ΔTM S.

Software, BioRad). Binding of S proteins to ACE2 (R&D Systems Inc.) was tested in a standard ELISA. In this assay, ACE2 served as a capture molecule and an anti-SARS S monoclonal antibody or anti-sera was used as a detection reagent. There was a linear relationship between the optical density and SARS S protein concentration in a range of 0.1–1.0 μg/ml. A second manuscript detailed in cloning, expression, purification, and characterization of the S proteins is in preparation.

2.2. Formulation

In the preparation of the vaccine, the ΔTM S protein was formulated in 20 mM sodium phosphate saline, pH 7.0 at four dose levels, 3, 9, 27, and 50 μg/50 μl, whereas the His-tagged full-length S glycoprotein was formulated at 9 μg/50 μl. The PBS used in the formulations served as a vehicle control (Table 1). In the follow-up study of vaccine with adjuvant (Alhydrogel®, Accurate Chemical and Scientific Corporation, Westbury, NY), ΔTM S protein was formulated in 2.5 mM sodium phosphate saline, pH 7.0, containing 1.6 mg aluminum/ml. In these preparations, ΔTM S was formulated at 1 μg/50 μl (±aluminum), 5 μg/50 μl (±aluminum), 50 μg/50 μl (−aluminum) and the His-tagged full-length S glycoprotein was formulated at 5 μg/50 μl. Alhydrogel in 2.5 mM PBS and PBS served as control (Table 2). Non-adjuvant vaccine preparations were stored at −20 °C, while adjuvanted vaccine preparations were stored at 2–8 °C.

2.3. Immunization

Both non-adjuvanted and adjuvanted recombinant S glycoproteins were tested in mice for immunogenicity, by IIT Research Institute (IITRI). The vaccine preparations were administered to male and female CD1 mice (VAF/Plus, Charles River Laboratories, Kingston, NY) by intramuscular (IM) injection according to the study designs shown in Tables 1 and 2. The animals were weighed and assigned to treatment groups using a constrained random process such that all groups tested were comparable in pre-test body weight, and the weight variation of each animal used would not exceed ±20% of the mean weight. The animals received 50 μl of the dose formulation containing graded doses of the two antigen preparations. On the days specified, animals were euthanized and sera collected.

2.4. Anti-SARS ΔTM S end point titers

Anti-ΔTM S titer of each serum was determined by ELISA. Briefly, a 96-well plate (NUNC-Immuno Maxi-Sorp) was coated with 1 μg/ml of purified ΔTM S protein in PBS and incubated overnight at 2–8 °C. Serial two-fold dilutions of mouse sera were prepared in a 96-well microtiter plate with antibody buffer, containing 150 mM NaCl, 0.3% Tween 20, 1% BSA, 5% non-fat dry milk, and 1% NaN_3 in 100 mM Tris–HCl, pH7.8. A 100 μl aliquot of each serum dilution was transferred to the respective well of the coated ELISA plate

Table 2
Mouse immunogenicity study with adjuvant aluminum hydroxide

Group	Test article	Dose (μg)	# Mice/day of blood sampling					
			Day 0[a]	Day 15[a]	Day 30[a]	Day 45[a]	Day 60	Day 75
1	ΔTM S	1	–	8	8	8	8	8
2	ΔTM S	1 + alum	–	8	8	8	8	8
3	ΔTM S	5	–	8	8	8	8	8
4	ΔTM S	5 + alum	–	8	8	8	8	8
5	FL His-tag	5	–	8	8	8	8	8
6	ΔTM S	50	–	8	8	8	8	8
7	Alhydrogel	–	–	4	4	4	4	4
8	Vehicle	–	4	–	4	–	–	4

[a] Dose day.

coated with ΔTM S protein. The plate was incubated at room temperature for 1 h. After washing with PBS, goat anti-mouse IgG peroxidase conjugate (Amersham-Pharmacia Biotech), diluted 1:2000 with the antibody buffer, was added to the wells of the plate. After a further 1 h incubation at room temperature, the plate was washed with PBS and a preparation of 3,3′,5,5′-tetramethylbenzidine (TMB, liquid substrate system for ELISA, Sigma) was added as a peroxidase substrate. The plate was incubated at 37 °C for 30 min, the color stabilized by adding 100 μl of 3N H_2SO_4 to each well, whose OD 450 was then monitored on a Multiskan EX plate reader. Serum samples were tested in singlet, and both positive and negative controls, pooled from eight mice, respectively, were also included in each ELISA plate. Geometric mean titer (GMT) of each dose at each bleed day was calculated based on titers of eight mice.

2.5. SARS-CoV-neutralization test

The virus neutralization (VN) test as described by [12] was performed. Vero-E6 cells were grown in 96-well cell culture plate until confluent monolayers formed. All sera were heated at 56 °C for 30 min before testing and assayed in duplicate. Sera were subjected to two-fold serial dilutions from 1:10 to 1: 5120 in a microtiter plate and 100 pfu of SARS-CoV strain Tor 2, P4 added to each well and the preparation incubated at 37 °C for 2 h, A human SARS-CoV convalescent serum and a non-immune human serum were used as positive and negative controls. After incubation, 100 μl of serum/virus mixture was transferred from each well of the incubation plate to a 96-well cell culture plate containing Vero-E6 cells and the plate incubated at 37 °C for 3 days. The plates were then evaluated for the presence of characteristic SARS-CoV cytopathic effect (CPE) [12]. A neutralization titer was defined as the reciprocal of the serum dilution in the well immediately before the well manifesting CPE. A GMT was calculated for each dose for each bleed day, based on the titers obtained from eight mice.

3. Results

3.1. Immunogenicity of ΔTM S vaccine without adjuvant

The first study was designed to test immunogenicity of the recombinant SARS-CoV ΔTM S glycoprotein vaccine in mice. Ecto-domain of SARS-CoV S glycoprotein, ΔTM S, was produced using a baculovirus-insect cell expression system in a serum-free cell line, *ExpresSF+*®, and purified to apparent homogeneity (manuscript in preparation). This protein was shown to bind to soluble ACE2 [18,19] (R&D Systems Inc.) in an ELISA assay (Zhou, unpublished result), demonstrating that it has the conformation required for functional binding to the receptor. The protein was formulated in 20 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl, at dose levels of 3, 9, 27, and 50 μg/50 μl. A 9 μg/50 μl preparation of His-tagged full-length SARS S glycoprotein was included in the assessment to provide an insight into differences in immunogenicity between the truncated and full-length proteins.

Groups of eight mice, equally male and female, were injected intramuscularly (IM) for a total of one, two, three or four doses every 15 days (study days 1, 15, 30, and 45) (Table 1). No animals died during the study and no test material-related adverse clinical signs were noted. All the animals gained weight, and there were no significant differences in body weight or body weight gain between the groups.

Sera were collected from all animals and tested for response to SARS-CoV by ELISA. A GMT was calculated based on serum titers of each group at each bleed day. GMTs from the study were summarized in Table 3. Both ΔTM S and His-tagged full-length SARS S glycoprotein were found to evoke a strong immune response. GMTs of mice immunized with 3, 9, and 50 μg preparations reached maximum antibody titers after three immunizations (Fig. 2A), while those immunized with 9 μg of the His-tagged S glycoprotein and 27 μg of the ΔTM S required four injections to reached maximum titers. The 9 μg His-tagged S glycoprotein group showed a similar titer range as that of the 27 μg group immunized with ΔTM S. Enhancement in immune response due to dose escalation was evident between the 3 (or 9) μg group and 27 (or 50) μg group, but not between 3 and 9 μg dose, or between 27 and 50 μg dose.

Sera were tested for SARS-CoV-neutralizing antibodies. To inactivate non-specific neutralizing factors all sera were heated to 56 °C for 30 min. GMTs of each vaccine group for each bleed day are summarized in Table 4. This VN test showed that antibodies elicited by ΔTM S and His-tagged full-length spike proteins are able to neutralize the SARS-CoV. In addition, the VN antibody GMT increases with increasing doses and antigen concentration. As shown

Table 3
ELISA GMTs of mouse anti-ΔTM S sera collected from the first mouse immunogenicity study without adjuvant

Group	Test article	Dose (μg)	GMT					
			Day 1	Day 15	Day 30	Day 45	Day 60	Day 75
1	ΔTM S	3	–	4666	43069	152219	98701	90510
2	ΔTM S	9	–	6727	45255	165995	98701	128000
3	FL His-tag	9	–	3175	19027	152515	279442	215689
4	ΔTM S	27	–	5187	98701	152219	279170	215269
5	ΔTM S	50	–	9514	139585	304437	234753	234753
6	Vehicle	–	<100	–	<100	–	<100	<100

Table 4
GMTs of virus neutralizing antibody of mouse anti-ΔTM S sera collected from the first mouse immunogenicity study without adjuvant

Group	Test article	Dose (μg)	GMT					
			Day 1	Day 15	Day 30	Day 45	Day 60	Day 75
1	ΔTM S	3	–	20	59	174	87	80
2	ΔTM S	9	–	14	67	72	87	135
3	FL His-tag	9	–	14	40	123	174	135
4	ΔTM S	27	–	10	87	98	226	207
5	ΔTM S	50	–	13	190	207	207	174
6	Vehicle	–	<10	–	<10	–	<10	<10

in Table 4, the GMT peaked after four injections except for 3 μg dose group, which was highest after three injections. There is a good correlation between antibodies measured by ELISA and VN antibodies (Tables 3 and 4), suggesting that the ELISA may be used as a "surrogate" to measure functional antibodies if SARS-CoV-neutralization test is not available.

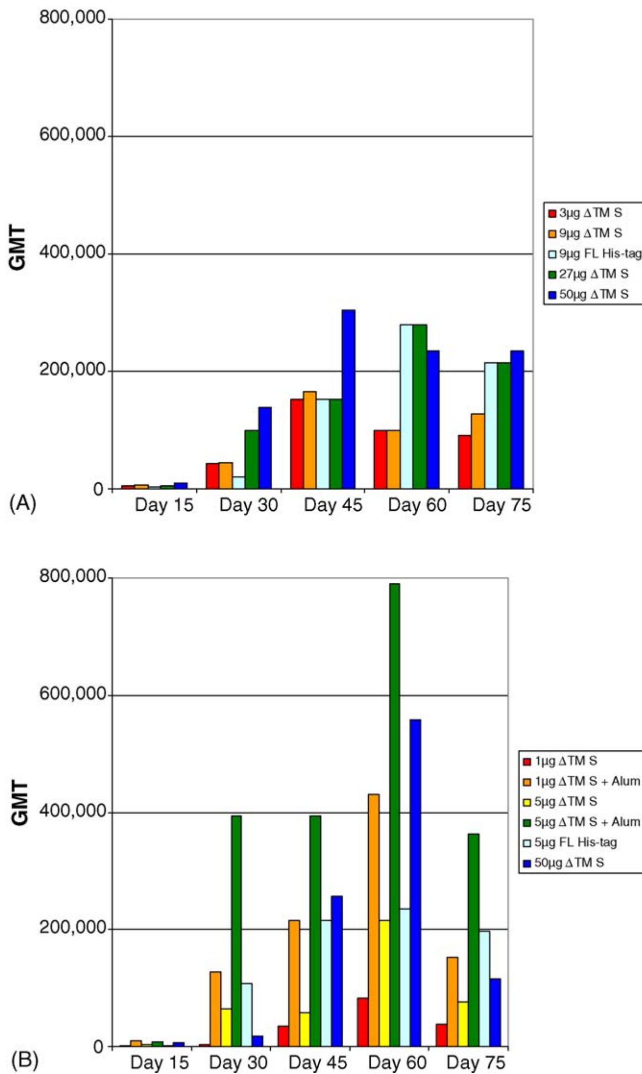

Fig. 2. (A) Immunogenicity in mice of SARS spike proteins without adjuvant. (B) Immunogenicity in mice of SARS spike proteins with aluminum hydroxide as adjuvant.

3.2. Immunogenicity using aluminum hydroxide as adjuvant

To evaluate whether the inclusion of an adjuvant into the SARS-CoV preparations would alter the ELISA and VN titers, a second mouse immunogenicity study was performed. Two preparations of ΔTM S at 1 and 5 μg/50 μl, were formulated with or without adjuvant, $Al(OH)_3$. Each adjuvanted dose (50 μl) contained 80 μg of aluminum, or 1.6 mg/ml, equivalent to 800 μg of aluminum in a 0.5-ml human dose. Due to competition of divalent cation phosphate for $Al(OH)_3$ binding, phosphate concentration of the formulation buffer was reduced from 20 mM (first study) to 2.5 mM. At this concentration, at least 95% of ΔTM S in 5 μg dose (5 μg/50 μl or 100 μg/ml) was adsorbed to aluminum hydroxide (80 μg/50 μl or 1.6 mg/ml) (data not shown). $AlPO_4$ ("ADJU-PHOS," Accurate Chemical and Scientific Corporation, Westbury, NY), was also evaluated for ΔTM S absorption and was found to be less efficient for aluminum hydroxide binding than $Al(OH)_3$ (data not shown).

Two non-adjuvanted preparations containing, 5 μg/50 μl of His-tagged full-length SARS S glycoprotein and 50 μg/50 μl of ΔTM S, were included to compare performance of non-adjuvanted preparations to those containing adjuvant.

Groups of eight mice, equally male and female, were injected intramuscularly (IM) for a total of one, two, three or four doses every 15 days (study days 0, 15, 30, and 45) (Table 2). In this study, one animal died and another suffered from weight loss. Both of the animals were in the high dose group (50 μg/50 μl). No other test material-related adverse clinical signs were noted. There were no significant differences in body weight or body weight gain between the groups. Additionally, no significant differences were observed in body temperatures 24 h following dosing. Therefore, it was determined that the described events were incidental and consistent with normal variations observed in mice and not biologically significant.

Sera from all the animals were collected. End point titers of the sera were determined by ELISA. GMTs were calculated for each group and the results are summarized in Table 5.

GMTs of all six dose groups peaked on Day 60 after four injections, and dose responses were clearly demonstrated (Fig. 2B). As observed in the first study, a limited immune response was observed across all the groups after

Table 5
ELISA GMTs of mouse anti-ΔTM S sera collected from the second mouse immunogenicity study with adjuvant aluminum hydroxide

Group	Test article	Dose (μg)	GMT					
			Day 0	Day 15	Day 30	Day 45	Day 60	Day 75
1	ΔTM S	1	–	2000	4000	34896	82998	38055
2	ΔTM S	1 + alum	–	9752	128000	215269	430539	152219
3	ΔTM S	5	–	2828	64000	58688	215269	76109
4	ΔTM S	5 + alum	–	8833	394806	394806	789612	362039
5	FL His-tag	5	–	2378	107635	215269	234753	197403
6	ΔTM S	50	–	7127	17500	256000	558340	115933
7	Alhydrogel	–	–	<100	<100	<100	<100	<100
8	Vehicle	–	<100	–	<100	–	–	<100

Table 6
GMTs of virus neutralization test of mouse anti-ΔTM S sera collected from the second mouse immunogenicity study with adjuvant

Group	Test article	Dose (μg)	GMT					
			Day 0	Day 15	Day 30	Day 45	Day 60	Day 75
1	ΔTM S	1	–	20	16	59	177	104
2	ΔTM S	1 + alum	–	20	238	390	761	381
3	ΔTM S	5	–	10	66	101	538	269
4	ΔTM S	5 + alum	–	25	226	698	1396	1174
5	FL His-tag	5	–	10	67	195	269	587
6	ΔTM S	50	–	32	57	247	293	640
7	Alhydrogel	–	–	<10	<10	<10	<10	<10
8	Vehicle	–	<10	–	<10	–	–	<10

primary immunization, i.e., a single dose does not appear to be adequate with or without the use of an adjuvant. Titers of mice receiving PBS as a control remained at baseline throughout the study. The adjuvanting effect of $Al(OH)_3$ was evident across all remaining time points following the second injection. Administration of adjuvanted vaccines resulted in GMTs several fold higher compared to corresponding non-adjuvanted vaccines. For instance, adjuvanted 1 μg ΔTM S group had titers similar to or higher than the non-adjuvanted 50 μg ΔTM S group, while 5 μg plus alum group had a GMT twice as high as the 50 μg group.

On the other hand, the 50 μg group had a slow immune response and titers peaked late. Nevertheless, the titer range was comparable with that of the first study. The 5 μg His-tagged S glycoprotein group also had a very comparable response to the His-tagged group in the first study (Fig. 2A).

All the mouse sera were tested for the development of SARS-CoV-neutralizing antibody. GMTs were calculated for each dose group at each time point and presented in Table 6. Highest GMT was observed after four injections in all vaccine groups. Titers of low dose ΔTM S groups (1 or 5 μg), with or without adjuvant, peaked on Day 60, while both His-tagged full-length (5 μg) and 50 μg ΔTM S groups showed highest titers at the last time point, Day 75. As previously observed in GMT measurements by ELISA, higher VN GMTs were apparent in the aluminum hydroxide adjuvant groups. Both adjuvant groups had titers several-fold higher than their corresponding non-adjuvant groups. Adjuvanted 1 μg ΔTM S dose elicited VN titers close to or higher than those observed in non-adjuvanted 50 μg ΔTM S group. Again, adjuvanted 5 μg ΔTM S dose resulted in a two-fold higher VN GMT than observed for the 50 μg group. The highest GMT of this dose group was 1:1396 at Day 60.

Overall, the recombinant protein vaccine ΔTM S was shown to be immunogenic in mice and to elicit SARS-CoV-neutralizing antibodies. This vaccine shows an increasing antibody response with increasing doses and antigen concentration. ΔTM S appears to perform equivalent to His-tagged full-length S glycoprotein in both studies. Adjuvanticity of aluminum hydroxide was clearly demonstrated at two different dose levels, 1 and 5 μg.

4. Discussion

A number of studies have shown that S protein and its fragments induce SARS-CoV-neutralizing antibodies [16,24–33]. The receptor-binding domain (RBD) in the S1 region plays a critical role in the neutralizing antibody induction as well as ACE2 binding and viral entry. Jiang and coworkers showed that RBD-Fc fusion induced high neutralizing antibody titers in mice, with a mean 50% titer of 1/15,360 against SARS-CoV infection [29]. Depletion of RBD-specific antibodies from sera significantly reduced serum-neutralizing capability [28], indicating this domain is dominant for the neutralizing antibody induction.

In this report both SARS S subunit vaccines, His-tagged full-length S and ΔTM S, contain RBD (Fig. 1A). With or without adjuvant, they were shown to be effective antigens in eliciting a strong neutralizing antibody response in mice. This is encouraging since highly purified proteins by themselves may be poorly immunogenic or non-immunogenic

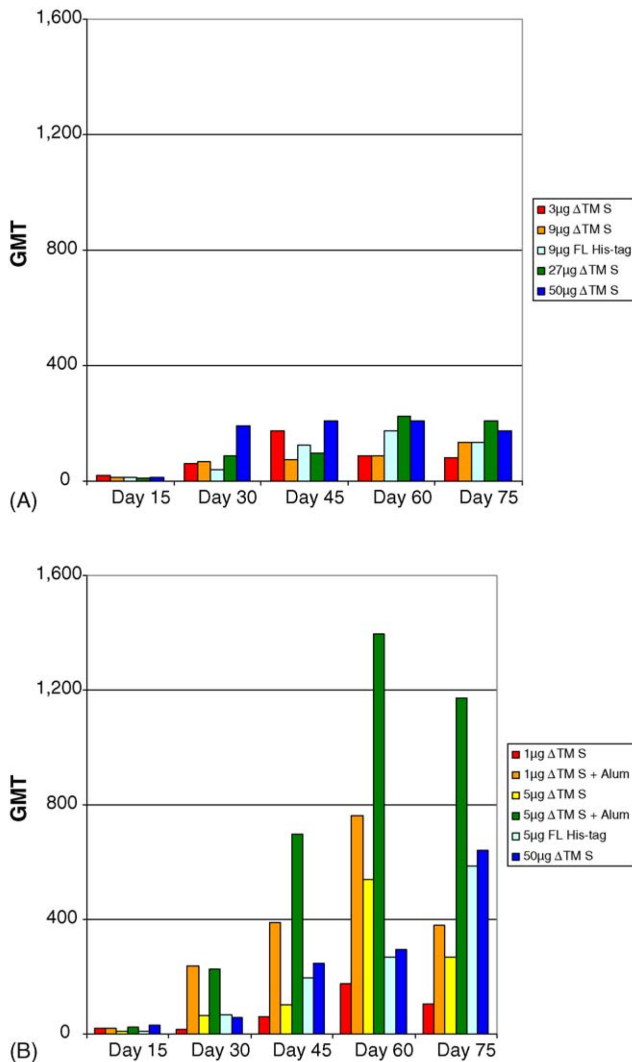

Fig. 3. (A) Viral neutralization titers of heat-treated anti-sera collected from mice immunized with SARS spike proteins without adjuvant. (B) Viral neutralization titers of heated anti-sera collected from mice immunized with SARS spike proteins with aluminum hydroxide.

[36]. The first pre-clinical study of the ΔTM S protein vaccine was conducted to address several questions, including safety, immunogenicity, ability to elicit SARS-CoV-neutralizing antibody, dose escalation, and comparison with His-tagged full-length S glycoprotein. Immune responses as determined by ELISA were strong, with GMTs as high as 300,000 and plateaued around 200,000 (Fig. 2A). SARS-CoV-neutralizing antibody titers averaged around 1:200 (Fig. 3A). Within each group the GMT increased with increased dose number or increased antigen concentration. While there was no clear difference observed between 3 and 9 μg doses, and the 27 and 50 μg doses, a trend is evident.

Groups (27 and 50 μg) had peak GMTs of 279,170 and 304,437 for ELISA, 226 and 207 for VN, respectively. These values are close even though the dosage in the later group nearly doubles, indicating that immune responses could not be improved further by simply injecting more protein. The question was raised whether an adjuvant could further enhance immunogenicity.

Aluminum hydroxide (Alum), introduced in the 1920's, has been frequently used as an adjuvant. It enhances immunogenicity by converting soluble protein vaccines into particulate mass to make them more suitable for ingestion by antigen-presenting cells such as macrophages [36]. The adjuvant potential of aluminum hydroxide was explored in the second mouse immunogenicity study. As shown in Fig. 3B, adjuvant effect was obvious in both 1 and 5 μg ΔTM S + Alum groups on Day 30 after the second administration. There were several fold differences in titer between adjuvanted and non-adjuvanted groups. Similar observations were obtained in VN GMT. The 1 μg adjuvanted dose induced an immune response equivalent to that of 50 μg non-adjuvanted dose, suggesting that formulation of ΔTM S with aluminum hydroxide results in a dramatic improvement in immune response. It was however observed that even with the inclusion of adjuvant, multiple doses were required to elicit a strong antibody response.

There appears to be a strong correlation between GMTs obtained with ELISA and those determined in the actual VN assay, suggesting that the ELISA may be used as a "surrogate" assay for the actual VN assay while investigating potential antigens and their immune responses. This is an important finding since the VN assay, which uses the live virus must to be performed under BSL-3 conditions and hence could be reserved for the final assessment.

The biological significance of the higher GMT observed while adjuvanting the antigen with Alum remains to be determined. We are planning to conduct a SARS-CoV challenging study in ferrets to study whether adjuvanting of the vaccine can indeed result in better protection against disease. Proof that the vaccine is effective in preventing disease in ferrets will provide valuable evidence to plan a Phase I clinical study to evaluate the immunogenicity of this recombinant protein vaccine in human subjects.

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