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Intranasal Protollin-formulated recombinant SARS S-protein elicits respiratory and serum neutralizing antibodies and protection in mice

Mary C. Hu [a], Taff Jones [b,*], Richard T. Kenney [c], Dale L. Barnard [d], David S. Burt [b], George H. Lowell [b]

[a] *GlaxoSmithKline Biologicals North America of Washington, 19204 North Creek Parkway, Bothell, WA 98011, USA*
[b] *GlaxoSmithKline Biologicals North America of Quebec, 525 Cartier Blvd. West, Laval, Montreal, Quebec H7V 3S8, Canada*
[c] *GlaxoSmithKline Biologicals North America of Maryland, 6996 Columbia Gateway Drive, Columbia, MA 21046, USA*
[d] *Institute for Antiviral Research, Department of ADVS, Utah State University, Logan, UT 84322, USA*

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Abstract

The feasibility of developing a prophylactic vaccine against SARS was assessed by comparing the immune responses elicited by immunizing mice with a recombinant SARS spike glycoprotein (S-protein) formulated with different adjuvants, given by different routes. In both young and aged mice, an intranasal Protollin-formulated S-protein vaccine elicited high levels of antigen-specific IgG in serum, comparable to those elicited by an intramuscular Alum-adsorbed S-protein vaccine. Serum antibodies were shown to be virus neutralizing. Intranasal immunization of young mice with the Protollin-formulated vaccine elicited significant levels of antigen-specific lung IgA in contrast to mice immunized with the intramuscular vaccine in which no antigen-specific lung IgA was detected. Following live virus challenge of aged mice, no virus was detected in the lungs of intranasally immunized mice, in contrast to intramuscularly immunized mice whose lung virus titers were comparable to those observed in control mice.

Keywords: SARS; Intranasal vaccine; Neutralizing antibody; Protection

1. Introduction

Severe acute respiratory syndrome (SARS) is an infectious disease that appeared spontaneously in China in late 2002, and spread rapidly to other parts of the world, in all probability by air travel. A new coronavirus (SARS-CoV) was identified to be the causative agent of SARS [1–3] and its genome sequence was published in 2003 [4–6]. The outbreak in 2002–2003 caused approximately 8000 SARS-related cases with a mortality rate of about 10%. This relatively high death rate coupled with the apparent ease of transmission of SARS-CoV, posed a serious public health threat and prompted considerable efforts to develop a safe and effective prophylactic vaccine for use in the event of a re-appearance of the disease.

Since its emergence, several approaches have been used to develop a SARS vaccine. An inactivated whole SARS virus which was sequentially inactivated by formaldehyde treatment and UV irradiation, was shown to stimulate neutralizing and protective antibody responses in mice immunized subcutaneously [7]. Another whole virus vaccine comprising formaldehyde-inactivated, polyethylene glycol (PEG)-precipitated SARS virus given intranasally to mice, was reported to induce virus-specific lung IgA and serum IgG [8]. However, most SARS vaccine development has focused on subunit vaccines using the SARS spike glycoprotein

* Corresponding author. Present address: MedImmune, 297 N. Bernardo Ave., Mountain View, CA 94043, USA. Tel.: +1 650 603 2617.
E-mail address: jonesd@medimmune.com (T. Jones).

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(S-protein) which has been shown to contain the virus' Receptor Binding Domain (RBD) as well as neutralizing epitopes, or fragments of the S-protein containing either the RBD or neutralizing epitopes [9]. For example, a recombinant baculovirus-expressed S-protein was shown to be capable of inducing high titers of neutralizing antibody when given intramuscularly to mice with or without Alum [10], and a recombinant S-protein fragment containing the RBD induced high titers of neutralizing antibody against SARS-CoV when used as a booster in mice which had been primed with DNA vaccines [11].

Infection with SARS virus is thought to be mediated by binding to angiotensin-converting enzyme 2 (ACE2), a surface protein found on lung alveolar epithelial cells and on vascular endothelium [12]. Thus, it would be highly desirable for an effective vaccine to induce immunity in both the respiratory tract as well as in serum, i.e. mucosal and systemic immunity. This laboratory's approach has been to attempt the induction of both by mucosal immunization of mice with a subunit vaccine formulated with the proprietary adjuvant Protollin—*Shigella flexneri* LPS non-covalently associated with major outer membrane proteins from *Neisseria meningitidis* (Proteosomes). In many pre-clinical studies, intranasal administration of vaccines comprising antigens formulated with Protollin have been shown to elicit potent mucosal as well as systemic immunity [13–16], while clinical trials of Protollin alone (as an intranasal vaccine against *Shigella* disease) showed that it was safe and well-tolerated in human volunteers [17]. Thus, provided it was successful in eliciting appropriate immune responses in pre-clinical studies, a Protollin-formulated S-protein vaccine would have the potential to be tested in clinical trials as a human SARS vaccine. This report documents pre-clinical feasibility studies in mice with intranasal Protollin-formulated S-protein and compares the responses elicited and their phenotype with those elicited by an intramuscular Alum-adsorbed S-protein vaccine.

2. Materials and methods

2.1. Vaccines

Recombinant S-protein was purchased from Protein Sciences Corporation (Meriden, CT). The protein was a truncated version of the S-protein from SARS Urbani strain with a deletion of the transmembrane and cytoplasmic domains at the carboxyl terminus, and was expressed in a baculovirus expression system and purified as previously described [10]. Development and GMP lots of Protollin containing approximately equal weights of Proteosomes and *S. flexneri* LPS (strain BS103) were prepared by diafiltration as previously described [16] and stored at −80 °C. Freshly thawed aliquots of S-protein and Protollin were mixed approximately 1 h prior to immunization of mice. Alum-formulated S-protein was prepared by adsorbing freshly thawed S-protein onto 0.5% (v/v) Alhydrogel (Accurate Chemicals, NY).

2.2. Animal protocols

All animal procedures were performed in a Canadian Council on Animal Care and Association for Assessment and Accreditation of Laboratory Animal Care accredited animal facility, and all animal protocols received prior approval from the facility's in-house Animal Care Committee. Animals were housed five per cage in HEPA filtered cages, and food and water were available *ad libitum*. With the exception of the challenge studies, all experiments were performed on 6–8-week-old female BALB/c mice. Challenge studies were performed on aged female BALB/c mice, all of which were approximately 12 months old.

2.3. Immunogenicity studies

On day 0, groups of 10 mice were immunized intranasally under light isoflurane anesthesia with 15 μL (7.5 μL per nare) of Protollin-formulated S-protein (containing 5 or 1 μg of Protollin and 10 μg of S-protein). Twenty-five microliters of Alum-formulated S-protein (10 μg of S-protein adsorbed onto 0.5%, v/v, Alum) was given to mice by intramuscular injection in a hind limb. Control groups were inoculated intranasally with either PBS, S-protein (10 μg) or Protollin (5 μg) alone. All mice received a second dose of vaccine or control 14 days after the initial inoculation and on day 21, mice were euthanized by CO_2 asphyxiation, exsanguinated, and lung lavage was performed [18]. Spleens were aseptically removed and processed for the *in vitro* restimulation of splenocytes and quantitation of released cytokines. Sera and lung lavage fluid were stored at −80 °C until assay as previously described [18].

2.4. Splenic cell culture and cytokine measurement

Aseptically harvested spleens from each mouse in a particular group were pooled and processed into single cell suspensions in minimal essential medium (MEM) and then incubated with different concentrations of S-protein in a modification of a method described previously [18]. The cytokines released into the cell culture supernatants following incubation, were measured by quantitative ELISA using OptEIA kits (BD Biosciences) as per the manufacturer's instructions.

2.5. ELISA

Antigen-specific serum IgG and lung lavage IgA were measured by ELISA. 96-well microtiter plates were coated with 0.5 μg/mL S-protein and the subsequent ELISA procedure performed as previously described [18]. IgG or IgA antibody concentrations in serum or lung lavage fluid from mice in immunogenicity studies were quantitated by reference to standard curves of IgG or IgA included on every plate, as previously described [18]. Data were expressed as geometric mean titers of the individual mouse results within a group, and the statistical significance of antibody concen-

trations between the different groups of mice was assessed by ANOVA analysis of log-transformed individual mouse data by Tukey–Kramer pairwise comparisons.

2.6. Challenge study

Only aged BALB/c mice were used for challenge studies [19]. On day 0, groups of 20 mice were immunized intranasally under light ketamine anesthesia with either 15 μL (7.5 μL per nare) of Protollin-formulated S-protein (10 or 30 μg of S-protein and 3 μg of Protollin), or intramuscularly in a hind limb with 25 μL of Alum-formulated S-protein (10 or 30 μg). Control groups were inoculated intranasally with either PBS, S-protein (30 μg) or Protollin (3 μg) alone. All mice received second and third doses of vaccine or control on days 14 and 35 following the initial inoculation. On day 21, blood was collected from all mice for measurement of serum IgG and neutralizing antibody. On day 42, seven mice from each group were euthanized as above, and blood and lung lavage fluids collected for assessment of antigen-specific serum IgG and neutralizing antibody, and lung IgA. Lung lavage fluid was assayed for antigen-specific IgA in duplicate at a 1:5 dilution and the mean OD from duplicate wells was reported for each sample. The remaining 13 mice from each group were bled and then challenged by intranasal inoculation of 5×10^4 $TCID_{50}$ SARS-CoV (Urbani strain) in a volume of 30 μL. Three days post-challenge, mice were euthanized and their lungs were harvested. One lung from each animal was homogenized for subsequent determination of lung virus titers and the other lung processed for histopathological examination.

2.7. Neutralizing antibody assay

The method was a modification of a previously published method [20]. Equal volumes of heat-treated sera at dilutions of 1:50 or 1:100 were incubated with 200 $TCID_{50}$ of virus at 37 °C for 1 h. Surviving virus in the mixtures was titered by cytopathic effect (CPE) assay. Serial dilutions were plated in quadruplicate and the assay was performed in triplicate on the same plate for each serum. For the CPE assay, 0.1 mL of a neutralization sample was added directly to the cell culture plate containing cells at the appropriate cell density (1×10^4 Vero 76 cells/well) plated the previous day in 96-well plates. An additional 0.1 mL of cell growth medium containing 4% fetal bovine serum was then added to each well, gently mixed and incubated at 37 °C for 6 days which had been previously determined to be the optimal time required to achieve full cytopathic effect in the non-treated infectivity controls when using virus at 200 $TCID_{50}$ units. The wells in the plate were then scored by visual observation for CPE or cytotoxicity using light microscopy. CPE was graded on a scale of 0–4, 0 indicating no cytopathic effect and 4 indicating complete cytopathic effect. Titers were then calculated using the Reed–Muench equation [21]. The inverse of the last dilution of serum which completely protected cells from CPE was considered the virus neutralization titer for the serum. Results were expressed as geometric mean titers calculated from the replicate results for all mice within a treatment group.

Infectivity control virus titrations ("virus back titration") to control for virus deterioration during the assays of the sera were included by mixing equal volumes of virus and MEM. These controls were then treated in the same manner as the sera containing samples.

2.8. Lung virus titer determination

One lung from each challenged mouse was homogenized, the tissue fragments allowed to settle, and varying dilutions of the supernatant fluids were assayed in triplicate for infectious virus in Vero 76 cells by CPE assay. Titers ($TCID_{50}$ values) were calculated using the Reed–Muench method [21].

3. Results

3.1. Immunogenicity of intranasal vaccine of Protollin-formulated S-protein

Young (6–8-week-old) BALB/c mice were immunized with 10 μg S-protein given intranasally by formulation with 1 or 5 μg of Protollin, or intramuscularly adsorbed onto 0.5% (v/v) Alum. Antigen-specific serum IgG and lung lavage IgA were measured by ELISA and the results are shown in Fig. 1.

Following two immunizations, S-protein alone did not elicit detectable antigen-specific serum IgG or lung IgA in intranasally immunized control mice. In contrast, intranasally administered S-protein formulated with Protollin induced high levels of antigen-specific serum IgG. The levels induced by formulation with 1 μg of Protollin were comparable to those induced by Alum-formulated S-protein ($P > 0.05$), while those induced by formulation of S-protein with 5 μg of Protollin were significantly higher than those elicited by the Alum-adsorbed S-protein ($P < 0.05$). Furthermore, both intranasal vaccines elicited levels of antigen-specific lung IgA significantly higher than background, while no specific IgA was detected in the lungs of mice given the intramuscular vaccine. The antigen-specific IgA response to the S-protein appeared highly dependent on the amount of Protollin in the formulation; S-protein formulated with 5 μg of Protollin elicited higher levels of IgA than S-protein formulated with 1 μg of Protollin.

Sera from the mice used in the immunogenicity studies were also assayed for neutralizing activity against the SARS-CoV. Both intranasal and intramuscular S-protein vaccines elicited detectable neutralizing antibody that was significantly higher than that observed in the control mice immunized with S-protein alone or PBS buffer (data not shown).

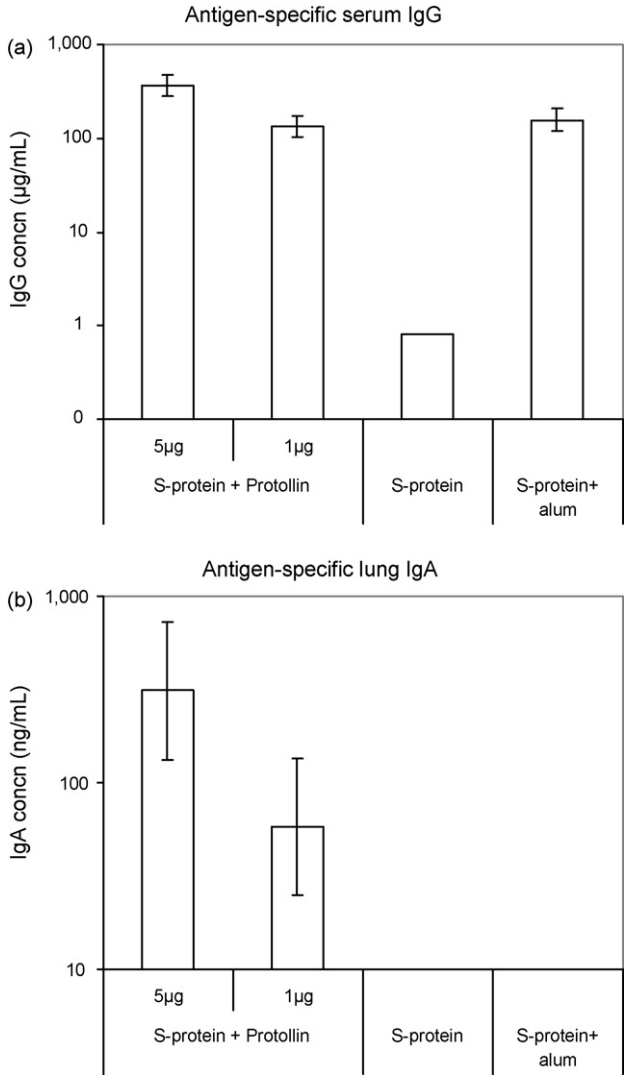

Fig. 1. Antigen-specific serum IgG (a) or lung IgA (b) concentrations in 6–8-week-old mice given 10 μg of S-protein either intranasally with Protollin (5 or 1 μg) or without, or intramuscularly adsorbed onto 0.5% (v/v) Alum. Geometric mean antibody concentrations (μg/mL for IgG, ng/mL for IgA) and 95% confidence limits for each group were calculated and reported.

3.2. Cytokine release from in vitro restimulated splenocytes

To assess the phenotype of the responses elicited by the intranasal or intramuscular vaccines, single cell suspensions of splenocytes from immunized mice were prepared and restimulated in culture by incubation with S-protein at different concentrations. The concentrations of the cytokines released into culture media were determined by ELISA.

As shown in Fig. 2, splenocytes from mice immunized intranasally with Protollin-formulated S-protein responded to *in vitro* restimulation by secreting IFN-γ, IL-2 and -6 at high concentrations, IL-4 at a moderate level and IL-5 at a low concentration. Compared with the intranasally immunized mice, splenocytes from mice immunized by intramuscular injection of Alum-adsorbed S-protein responded to *in vitro*

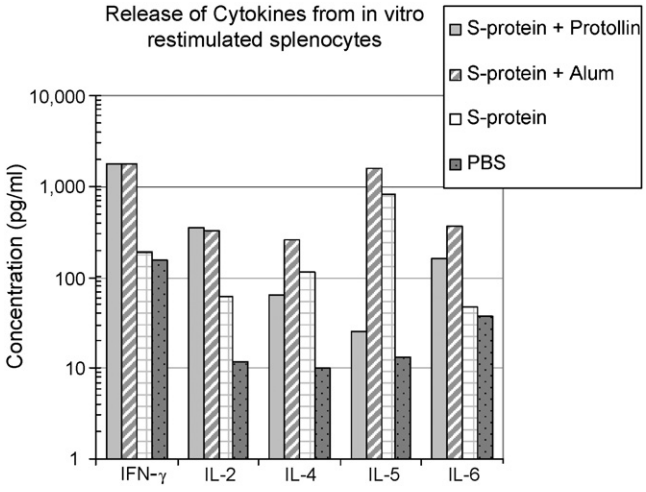

Fig. 2. The cytokines released into culture medium after mouse splenocytes were restimulated *in vitro* with S-protein for 48 h. Splenocytes were harvested from groups of mice immunized with 10 μg of S-protein with or without Protollin (5 μg), or adsorbed onto 0.5% (v/v) Alum. Control mice received PBS.

restimulation by secreting similar levels of IFN-γ and IL-2, IL-4 and IL-6 at levels about 3-fold higher, and most strikingly IL-5 at a concentration several hundred fold higher. Thus, the cytokine profile elicited by the intranasal Protollin-formulated S-protein vaccine was consistent with a balanced Th1/Th2 response or a response slightly biased towards a Th1 phenotype, whereas the cytokine profile induced by intramuscular injection of Alum-adsorbed S-protein was more consistent with a response biased towards a Th2 phenotype.

3.3. Challenge study

Groups of 20 aged mice (∼12-months) were immunized intranasally with Protollin-formulated S-protein or intramuscularly with Alum-adsorbed S-protein. Mice were immunized on days 0, 14, and 35, and sera was collected on days 21 and 42. Antigen-specific IgG in sera was determined by ELISA and the results are shown in Fig. 3a. Mice which were given 30 μg of unformulated S-protein intranasally generated detectable antigen-specific serum IgG titers after two immunizations, responses which were enhanced following a third immunization. However, both Protollin-formulated and Alum-adsorbed S-protein vaccines elicited significantly higher antigen-specific antibody responses compared with the unformulated S-protein ($P < 0.05$) on both days 21 and 42. It was noticeable though that even after three immunizations with either vaccine, the antigen-specific serum IgG titers elicited in the aged mice were approximately 3- or 4-fold lower than the serum IgG responses achieved by immunizing young mice with only two doses of the same vaccines. No antigen-specific IgG was detected in the serum of control mice which received either PBS buffer or Protollin alone.

The day 42 sera were also tested for virus neutralizing antibody. As shown in Table 1, both intranasal and intramuscular

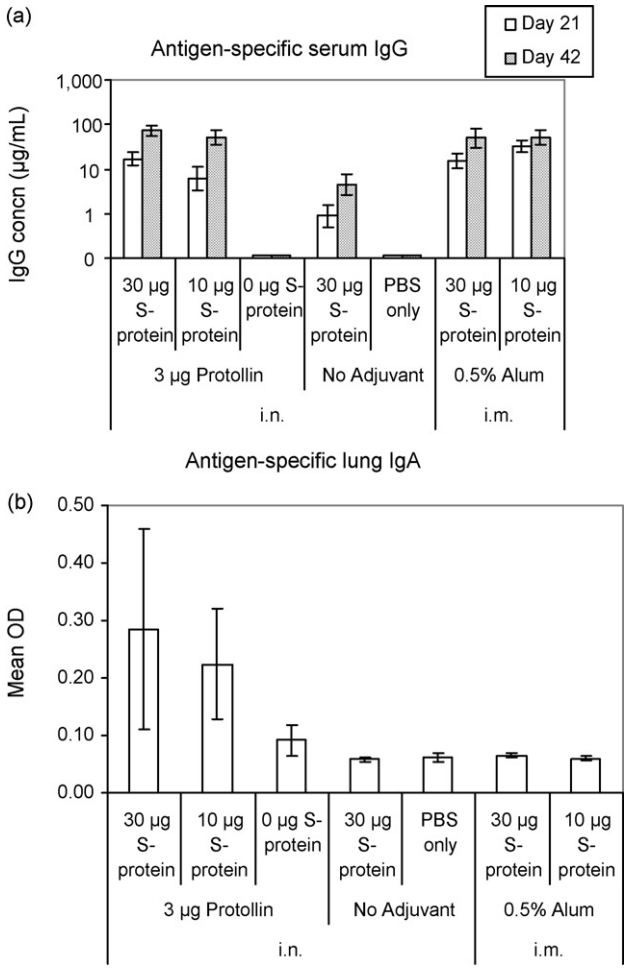

Fig. 3. Antigen-specific serum IgG (a) or lung IgA (b) concentrations in aged mice given 10 or 30 μg of S-protein either intranasally formulated with Protollin (3 μg) or intramuscularly adsorbed onto 0.5% (v/v) Alum. Control mice were given Protollin (3 μg) alone, S-protein (30 μg) alone or PBS intranasally. Serum IgG was calculated as geometric mean antibody concentration (μg/mL) and 95% confidence limits for each group were calculated and reported. Lung IgA concentrations were below the assay's limit of quantitation and the means of the recorded ODs are reported.

vaccines elicited similar levels of virus neutralizing antibody, which were higher than those in the control groups.

On day 42 prior to challenge, 7 mice from each group were euthanized and lung lavage was collected for assessment of antigen-specific IgA. Lung lavage was assayed in duplicate at a 1:5 dilution and the mean OD from duplicate wells was reported for each sample. Group means were calculated and are shown in Fig. 3b. Antigen-specific IgA at levels significantly above background was only detected in mice given Protollin-formulated S-protein. In all other groups of mice, OD's were indistinguishable from background.

On day 42, the remaining mice from each group were challenged by intranasal inoculation with live SARS virus (Urbani strain). Three days post-challenge, all mice were euthanized and one lung was homogenized for determination of virus titer and the other lung processed for histopathological examination. No significant histopathology findings were reported in any of the challenged mice. Virus titer measurements are reported in Table 1. Mice immunized intranasally with Protollin-formulated S-protein had significantly lower virus titers than those immunized intramuscularly with the Alum-adsorbed S-protein. None of the mice immunized with 30 μg S-protein formulated with Protollin had detectable virus titer in the lung while those given the lower 10 μg dose of antigen had detectable titers, albeit at levels 10-fold lower than the titers in the control mice given saline or adjuvant alone.

4. Discussion

The studies reported here were initiated to test the feasibility of developing an intranasal vaccine against SARS by comparing the responses elicited by a Protollin-formulated S-protein vaccine administered intranasally to mice with those elicited by an injected Alum-adsorbed S-protein vaccine. As expected from previous studies with the proprietary adjuvant Protollin [13–15], the data from the immunogenicity studies confirmed that after two doses of either vaccine, only the intranasal vaccine was capable of inducing antigen-specific responses in the mucosal compartment (i.e. antigen-specific IgA in lung lavage fluid) while both vaccines elicited comparable systemic responses. Also as in previous studies, the phenotype of the response was that of a balanced Th1/Th2 for the intranasal vaccine whereas the injected vaccine elicited a response more biased towards a Th2 phenotype.

To better understand the significance of the lung IgA responses, a challenge study was conducted in the aged mouse model [19]. This model has been proposed for SARS chal-

Table 1
The geometric mean titers (GMTs) of SARS virus neutralizing antibody in sera collected on day 42 (prior to challenge), and the within group means of lung virus titers on day 3 post-challenge

Adjuvant	Protollin				PBS control	Alum	
	3 μg	3 μg	3 μg	0 μg	0 μg		
S-protein	30 μg	10 μg	0 μg	30 μg	0 μg	30 μg	10 μg
Immunization route			Intranasal			Intramuscular	
GMT of neutralizing serum antibody	546	284	35	146	47	533	586
Group mean of lung virus titer	0.00	0.46	4.32	2.42	4.79	3.68	1.79

For neutralizing antibodies, each serum sample was assayed in duplicate and the GMT for each group was calculated. For virus titer measurement, individual lung homogenates were tested in duplicate and the group mean was calculated.

lenge studies as it may mimic the clinical observations from the 2002 to 2003 SARS epidemic when it was observed that advanced age was both an independent correlate of poor outcome and a predictor of mortality. Since it has been reported that the SARS virus can replicate in mice, the authors proposed the aged mouse as a model and demonstrated its usefulness in their hands [19].

For the challenge studies, 12-month-old BALB/c mice were immunized three times either intranasally with Protollin-formulated S-protein or intramuscularly with Alum-adsorbed S-protein. Both vaccines elicited comparable levels of antigen-specific serum IgG though the titers achieved by three immunizations fell short by a factor of 3 or 4 of the titers achieved by two immunizations of 6–8-week-old mice with the same vaccines. This however is not surprising given the well-documented effects of aging on the humoral immune system [22]. Antigen-specific lung IgA titers were also lower than those elicited in the 6–8-week-old mice, and were below the accepted ELISA's lower limit of quantitation. However, when OD values in minimally diluted lung lavage were used instead of titer analyses for the measurement of lung IgA, significantly higher S-protein-specific IgA levels were detected in the mice immunized intranasally with Protollin-formulated S-protein than in those immunized intramuscularly with Alum-adsorbed S-protein.

Although the specific serum IgG levels in aged mice were not as high as in young mice, sera collected after the third immunization and pre-challenge, were shown to possess strong neutralizing activity against live SARS virus. Furthermore, measurement of virus titers in the lungs of the challenged mice showed that there were much lower virus counts in the lungs of mice given the Protollin-formulated vaccine compared with the titers in the lungs of mice given the Alum-adsorbed vaccine or any of the control treatments. In fact none of the mice immunized intranasally with the higher 30 μg dose of S-protein formulated with Protollin showed any detectable virus titer in the lung. Thus, there was a qualitative correlation between the level of IgA in lung lavage fluid of intranasally immunized mice and virus titer recovered from their lungs following subsequent challenge. The fact that mice immunized with the Alum-adsorbed S-protein also developed high levels of serum neutralizing activity but no detectable lung IgA but had significantly higher viral lung titers than the intranasally immunized mice strongly supports the concept that specific lung IgA titers are an important and, in the case of SARS, perhaps potentially critical component of protection afforded by immunization with the Protollin-formulated, intranasal S-protein vaccine.

These studies have demonstrated that an intranasal vaccine comprising Protollin-formulated S-protein was clearly better at protecting mice in an aged mouse challenge model of SARS infection than an intramuscular Alum-adsorbed S-protein vaccine. These data indicate that further development of an intranasal Protollin-formulated S-protein vaccine to protect against the re-emergence of SARS disease is warranted. Such studies would include advanced development of antigen and adjuvant production, performance of pre-clinical toxicology and non-human primate studies, and early phase clinical trials designed to assess the vaccine's safety and immunogenicity.

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