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Virology 334 (2005) 160–165

VIROLOGY

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Rapid Communication

Neutralizing antibody and protective immunity to SARS coronavirus infection of mice induced by a soluble recombinant polypeptide containing an N-terminal segment of the spike glycoprotein

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Received 3 January 2005; returned to author for revision 26 January 2005; accepted 31 January 2005

Abstract

A secreted, glycosylated polypeptide containing amino acids 14 to 762 of the SARS coronavirus (SARS-CoV) spike protein and a polyhistidine tag was expressed in recombinant baculovirus-infected insect cells. Mice received the affinity-purified protein with either a saponin (QS21) or a Ribi (MPL + TDM) adjuvant subcutaneously and were challenged intranasally with SARS-CoV. Both regimens induced binding and neutralizing antibodies and protection against SARS-CoV intranasal infection. However, the best results were obtained with QS21 and protein, which provided the highest antibody as well as complete protection of the upper and lower respiratory tract.
Published by Elsevier Inc.

Keywords: Polypeptide; Glycoprotein; Respiratory tract

Introduction

Identification of the etiologic agent of severe acute respiratory syndrome (SARS) has made it possible to work toward the development of vaccines and therapeutics that could prevent future recurrences of the disease. SARS-coronavirus (SARS-CoV) has a nearly 30,000 nucleotides long RNA genome with 11 open reading frames that encode four major structural proteins consisting of nucleocapsid, spike (S), membrane, and small envelope protein (Marra et al., 2003; Rota et al., 2003). The S protein of SARS-CoV is a 180- to 200-kDa type-I transmembrane glycoprotein that has 20–27% amino acid identity with the corresponding protein of other CoVs, but unlike some is not cleaved into S1 and S2 subunits. The SARS-CoV S protein mediates cell entry by binding to a cell receptor identified as angiotensin-converting enzyme 2 (Li et al., 2003). An additional SARS-CoV receptor, CD209L, which also binds S has recently been identified (Jeffers et al., 2004). S is a target of neutralizing antibodies (Berry et al., 2004; He et al., 2004; Sui et al., 2004) making it an important candidate for vaccine applications. Currently studied vaccine candidates for which some protection has been demonstrated in animal models include DNA (Yang et al., 2004), modified vaccinia virus Ankara (MVA) (Bisht et al., 2004), and parainfluenza virus type 3 (Bukreyev et al., 2004) vectors that express S. Although recombinant DNA and expression vectors generally induce good cell mediated immunity, they frequently do not induce as a high an antibody response as adjuvanted proteins. Here, we demonstrate the induction of neutralizing antibody and protective immunity to SARS-CoV infection of mice induced by a soluble secreted polypeptide containing amino acids 14 to 762 of the S protein in combination with the saponin QS21 or the Ribi adjuvant MPL + TDM composed of monophosphoryl lipid A and trehalose dicorynomycolate. Both adjuvant types have been tested in phase I clinical trials of candidate vaccines against cancer and infectious diseases.

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0042-6822/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2005.01.042

Results

We considered it impractical to isolate the natural membrane-bound S glycoprotein from SARS-CoV and instead used a baculovirus/insect cell system to express an N-terminal fragment of S (nS) as a secreted glycosylated protein that could be readily purified under native conditions. The N-terminal 762 amino acids of the S protein was selected on the basis of hydrophilicity and secondary structure predictions using Kyte and Dolittle and Chou Fasman algorithms (McVector 7.2) and because it includes the region corresponding to S1 of other coronaviruses. A transfer vector was constructed in which the polyhedrin promoter regulates expression of a protein comprised of amino acids 14 to 762 of S preceded by the honeybee melittin signal peptide and followed by six histidines (Fig. 1A). A baculovirus expressing nS was derived by recombination in insect cells. The yield of secreted and affinity purified nS was approximately 10 mg/l of culture supernatant, and a single major band of ~110 kDa was seen by SDS-polyacrylamide gel electrophoresis after staining with Coomassie Blue (Fig. 1B, lane 1) or silver nitrate (Fig. 1B, lane 2). Upon Western blotting, the same 110-kDa band was recognized by antibodies to the polyhistidine tag and SARS-CoV S protein (Fig. 1B, lanes 3 and 4). Treatment with peptide N-glycosidase F reduced the mobility of the protein to ~85 kDa, demonstrating that the higher than expected apparent mass was due to N-glycosylation (Fig. 1C).

To analyze immunogenicity, nS protein mixed with MPL + TDM or QS21 adjuvant was injected subcutaneously into BALB/c mice on days 0, 28, and 56. Control mice were immunized with adjuvant and a secreted form of the vaccinia virus membrane protein L1R that was also produced in the baculovirus system and purified by affinity chromatography (Fogg et al., 2004). As an initial evaluation of immunogenicity, sera from the mice were tested for antibodies that recognize S protein expressed on the surface of cells by recombinant modified vaccinia virus Ankara (MVA/S) (Bisht et al., 2004). Because the endoplasmic reticulum acts as a filter for misfolded proteins, S present on the cell surface is likely to be correctly folded. Although SARS-CoV-infected cells could be used for the same purpose, considerably higher containment levels would be required. Uninfected HeLa cells or HeLa cells infected with non-recombinant MVA or MVA/S were fixed and stained with pooled mouse serum followed by Alexa 594-conjugated-anti-mouse IgG and analyzed by confocal microscopy. The serum obtained from mice immunized with nS in QS21 or MPL + TDM adjuvant stained the surface of cells infected with MVA/S but did not detectably stain uninfected cells or cells infected with non-recombinant MVA (Fig. 2). In contrast, serum from control mice that were immunized with the vaccinia virus L1R protein stained cells infected with non-recombinant and MVA/S equally (not shown). These data indicated that the antibodies produced by nS were able to bind to the membrane-associated form of full-length S.

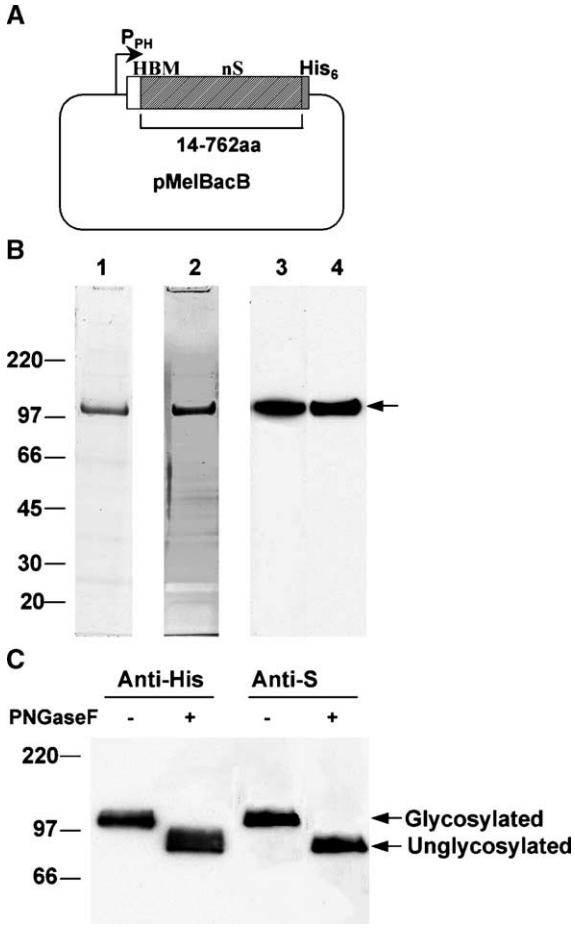

Fig. 1. Expression and characterization of SARS-CoV nS glycoprotein. (A) Schematic representation of pMelBacB-based baculovirus transfer vector. Abbreviations: P_{PH}, polyhedrin promoter; HBM, DNA encoding honeybee melittin signal sequence; nS, DNA segment encoding amino acids (aa) 14–762 of the SARS-CoV S protein; His_6, DNA encoding 6 histidine residues. (B) Purified nS analyzed by SDS polyacrylamide gel electrophoresis and Coomassie Blue staining (lane 1), silver staining (lane 2) and Western blot analysis with anti-His mAb (lane 3) or anti-SARS CoV S polyclonal antibody (lane 4). (C) Purified nS protein was (+) or was not (−) treated with peptide N-glycosidase F and was analyzed by SDS polyacrylamide gel electrophoresis and western blotting with anti-His mAb and anti-SARS-CoV S polyclonal antibody. Molecular masses of marker proteins in kDa are shown on the left.

The relative binding activity of pooled serum from mice immunized with nS and QS21 or MPL + TDM adjuvant was analyzed using nS as the capture antigen. Antibody was detected after the primary inoculation of nS with QS21 and the reciprocal ELISA titer was boosted to 1:409,600 after two more inoculations (Fig. 3A). With MPL + TDM adjuvant, the antibody response to nS was detected only after boosting but subsequently reached approximately 25% of the level achieved with QS21. The IgG2a/IgG1 ratio is an indicator of Th1 help. The specific IgG2a/IgG1 titers from mice immunized with QS21 and MPL + TDM were 0.25 and 0.03, respectively, suggesting a greater Th1 response with the former adjuvant. A determining effect of adjuvant on helper T cell responses has been noted (Cribbs et al., 2003; Santos et

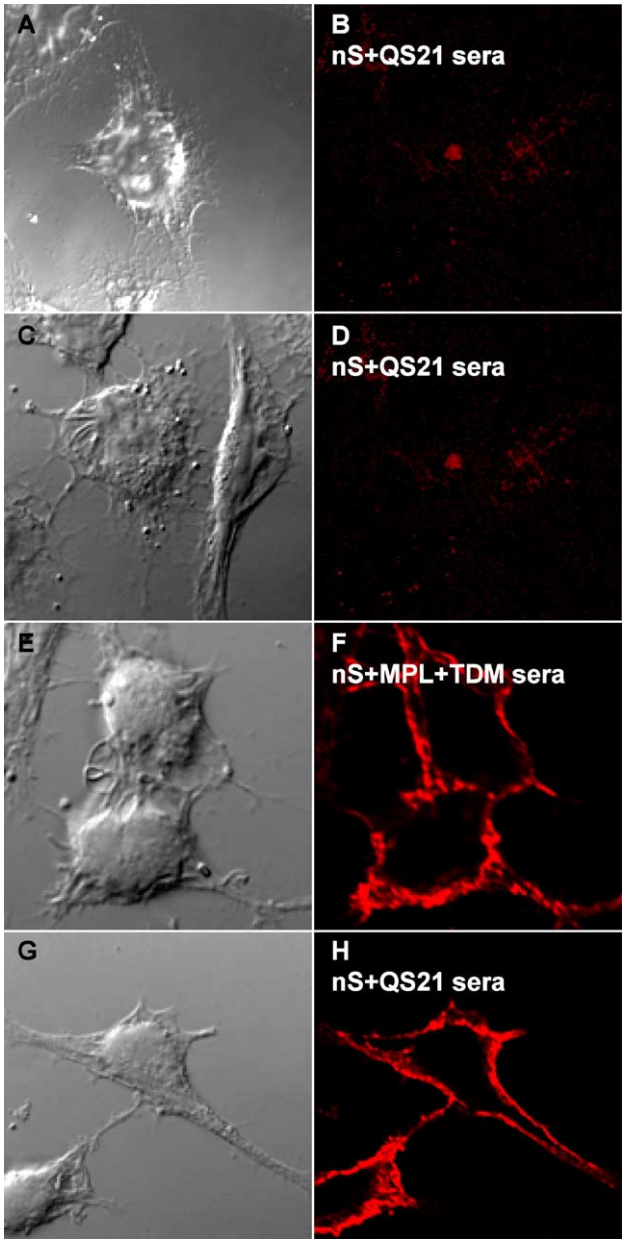

Fig. 2. Binding of antibodies from mice immunized with nS to full-length membrane-bound S. HeLa cells were uninfected (A–B), infected with non-recombinant MVA (C–D) or MVA expressing S (E–H) for 18 h. After fixation, the unpermeabilized cells were stained with pooled sera from mice immunized three times with nS and MPL + TDM (E–F) or nS and QS21 (A–D, G–H) followed by Alexa 594-conjugated anti-mouse IgG and viewed by visible (A, C, E, G) or fluorescence (B, D, F, H) light microscopy.

al., 2002). For comparative purposes, we also determined the IgG2a/IgG1 ratio of serum previously obtained from mice immunized with MVA/S. Although the overall IgG titers were lower in mice immunized with MVA/S (Bisht et al., 2004) than with the nS protein, the IgG2a/IgG1 ratios were higher with values of 2 and 4 for pooled sera of mice immunized intranasally and intramuscularly, respectively.

The high titer of nS-binding antibody and its recognition of full-length membrane-bound S encouraged us to evaluate the ability of the immune sera to neutralize the infectivity of SARS-CoV. Significant neutralizing activity was observed after the second inoculation of nS with either adjuvant (Fig. 3B). However, the mean neutralizing titer of 1:1269 achieved with QS21 was 4.6-fold higher than that obtained with MPL + TDM. Thus, there was good correspondence between the relative binding and neutralizing activities of sera obtained with QS21 and MPL + TDM adjuvants.

Subbarao et al. (2004) demonstrated that SARS-CoV replicates in the respiratory tract of BALB/C mice and that replication was reduced following passive administration of neutralizing antibody. In this model, peak titers were reached within 1 to 2 days depending on the dose and clearing occurred by 7 days. Two days after the intranasal

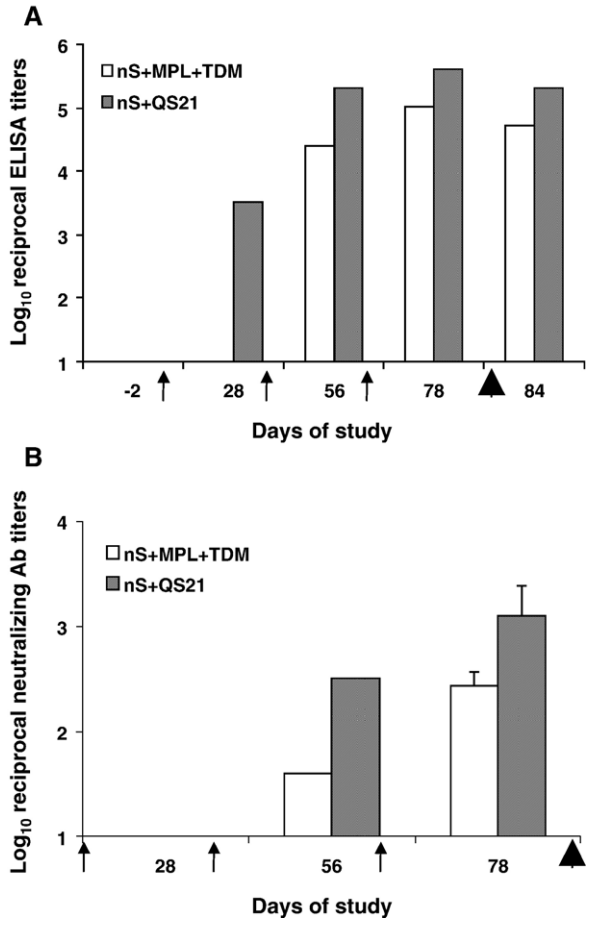

Fig. 3. ELISA and neutralizing antibody responses to nS. Groups of 7 BALB/c mice were immunized subcutaneously with 10 μg of purified nS and QS21 or MPL + TDM adjuvant at 4-week intervals (arrows) and challenged intranasally with 10^5 $TCID_{50}$ SARS-CoV on day 82 (arrow head). Control mice were immunized at the same times with purified soluble vaccinia virus L1R protein. (A) End-point ELISA titers of pooled sera collected on days indicated were measured using nS as the capture antigen. The absorbance obtained with serum from mice immunized with L1R was subtracted. (B) Dilution of serum that completely prevented cytopathic effects of SARS-CoV in 50% of wells containing Vero cells was calculated. Assays were performed on pooled serum collected on days 28 and 56 days and on individual mouse serum collected on day 78. Standard error bars are shown for the latter.

administration of 10^5 $TCID_{50}$ of SARS-CoV, 10^8 $TCID_{50}$ of virus per g of lung was recovered in control mice immunized with the vaccinia virus L1R protein in either adjuvant (Fig. 4A). By contrast, there was at least a 10^6-fold reduction in viral load in the lungs of mice immunized with nS regardless of the adjuvant (Fig. 4A). The difference was highly significant ($P = 0.0017$) as determined using the Mann–Whitney non-parametric statistical method. Indeed, virus was detected in only one mouse out of seven in each of the test groups.

The virus titers in the nasal turbinates showed a 10^3-fold reduction relative to controls when nS was administered with MPL + TDM adjuvant and $>10^4$-fold reduction when nS was given with QS21 (Fig. 4B). The effect of vaccination with either adjuvant was highly significant when compared with controls ($P = 0.0017$) determined as above. Virus was detected in the nasal turbinates of 4 of 7 test mice immunized with nS and the MPL + TDM adjuvant, whereas the titers were uniformly below detection in the turbinates of mice immunized with nS and QS21. The better protection obtained with the QS21 adjuvant was also statistically significant ($P = 0.0250$), using the Mann–Whitney non-parametric statistical method corrected for ties, consistent with the higher binding and neutralizing antibody titers. The failure of the nS antibody response to be boosted after challenge (Fig. 3A) was also consistent with the absence of virus replication.

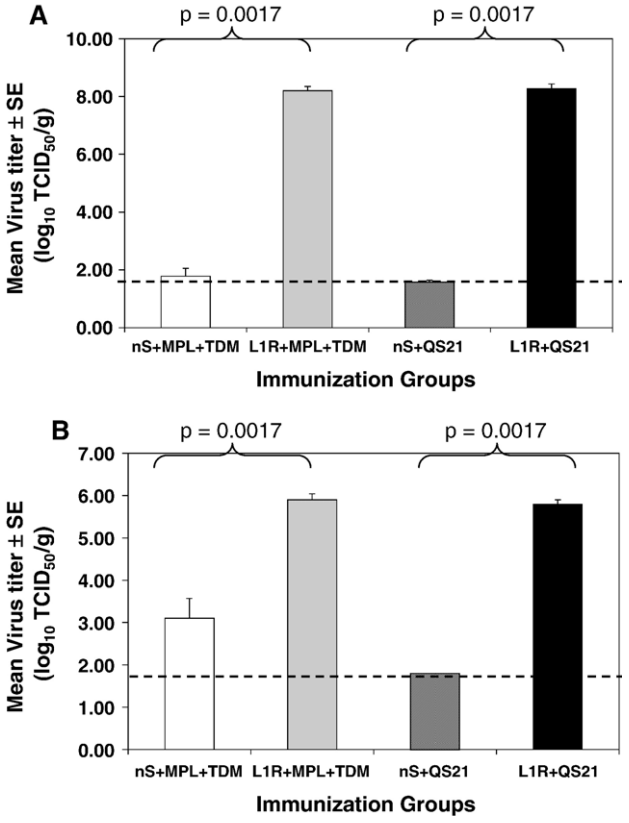

Fig. 4. Protection against SARS-CoV replication in immunized mice. Groups of 7 BALB/c mice were immunized and challenged with SARS-CoV as described in the legend to Fig. 3. Two days after the challenge, the virus titers (mean $\log_{10}TCID_{50}$ per g tissue with standard error) were measured in the lower (A) and upper (B) respiratory tract. The dotted line represents the lower limit of detection corresponding to 1.8 $\log_{10}TCID_{50}$ per g tissue for upper respiratory tract (A) and 1.5 $\log_{10}TCID_{50}$ per g tissue for lower respiratory tract (B).

Discussion

A recombinant polypeptide containing amino acids 14 to 762 of the SARS-CoV S protein administered with adjuvant induced neutralizing antibody and protectively immunized mice against upper and lower respiratory infections with SARS-CoV. Although the ability of a protein vaccine to protectively immunize against SARS-CoV was not previously reported, recent studies have shown that the protein segment we used contains the angiotensin-converting enzyme 2 receptor-binding region (Babcock et al., 2004; Wong et al., 2004; Xiao et al., 2003) as well as immunodominant and neutralizing epitopes (He et al., 2004; Lu et al., 2004; Sui et al., 2004; Zhou et al., 2004). The protein vaccine induced higher neutralizing antibody and more complete protection against an intranasal SARS-CoV challenge than that achieved by inoculation of mice with live SARS-CoV (Subbarao et al., 2004), MVA expressing the full-length S (Bisht et al., 2004), or DNA expressing full-length S or S lacking the transmembrane and cytoplasmic domains (Yang et al., 2004). The better protection achieved in this study is correlated with the higher antibody response. Although nS with either QS21 or MPL + TDM was effective, the former adjuvant induced higher binding and neutralizing antibody and better protection of the upper respiratory tract. Vaccination with QS21 also induced a more balanced helper T-cell response than MPL + TDM as indicated by the higher IgG2a/IgG1 ratio. However, we attribute the greater protection with QS21 adjuvant to the higher overall antibody response since MVA/S induced a considerably higher IgG2a/IgG1 ratio but was less protective than nS with QS21.

The mouse model of SARS-CoV has been used in previous vaccine studies and therefore allowed us to compare those results with the present ones. However, the mouse model has limitations because of the absence of disease and the relatively short period of replication (Subbarao et al., 2004). Clinical illness has been reported in some monkey studies (Kuiken et al., 2003) but not in others (Bukreyev et al., 2004; McAuliffe et al., 2004). SARS-CoV has a relatively long period of replication in ferrets (Martina et al., 2003) and Weingartl et al. (2004) recently reported that a recombinant MVA expressing S did not protect ferrets but contributed to hepatitis following challenge with SARS-CoV. In that study, neutralizing antibody was produced only transiently following immunization and was not detectable at the time of challenge although it was subsequently boosted. In another study, ter Meulen et al. (2004) showed that passively administered antibody reduced the replication of SARS-

CoV in the lungs of ferrets. It will be interesting to determine whether a protein subunit vaccine is capable of high and sustained antibody in ferrets or other animals that are highly susceptible to SARS-CoV.

Materials and methods

Vector construction

A cDNA encoding amino acids 14 to 762 of the SARS-CoV (Urbani strain) S protein (GenBank accession no. AY278741) with 6 histidine residues appended to the C-terminus was inserted into the *Bam*HI and *Eco*RI sites of the baculovirus transfer vector pMelBacB (Invitrogen) so that the honeybee melittin signal peptide was in frame with the S protein. The plasmid and linearized *Autographa californica* multiple nuclear polyhedrosis virus DNA were transfected into SF9 and a recombinant baculovirus was clonally purified following the Bac-N-Blue system protocol (Invitrogen).

Expression and purification of recombinant nS protein

High Five cells were infected with recombinant baculovirus at a multiplicity of infection of 10 for 120 h at the National Cell Culture Center (Minneapolis, MN) and the culture medium was frozen and shipped to us. The culture supernatant was concentrated 5-fold with a Millipore Labscale transverse flow filter system and was clarified by centrifugation in a Sorvall H6000A rotor at 3000 rpm for 30 min at 4 °C. The supernatant was dialyzed against phosphate pH 7.4 buffered saline (PBS) and then incubated with a 50% (wt/vol) slurry of nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) for 3–4 h at 4 °C. The mixture was loaded into a column that was washed with 10 bed-volumes of wash buffer (50 mM phosphate pH 8 buffer/300 mM NaCl/10 mM imidazole/1 mM phenyl methyl sulfonyl fluoride), 10 bed-volumes of wash buffer containing 25 mM imidazole, 2 bed-volumes of wash buffer containing 40 mM imidazole, and 3 bed-volumes of wash buffer containing 200 mM imidazole. The pooled 200 mM imidazole eluate containing nS was dialyzed against PBS and concentrated using a Millipore Amicon ultra filter. Protein samples were analyzed on a 4–12% bis-Tris polyacrylamide gel (Invitrogen) and stained with GelCode Blue stain reagent (Pierce) and with Silver Stain Plus kit (BioRad). Where indicated, N-glycosidase F treatment was carried out as previously described (Bisht et al., 2004).

Immunological assays

Western blotting was carried out as previously described using anti-His mouse mAb (Qiagen) or anti-SARS-CoV S rabbit polyclonal antibody (IMG-541, Imgenex, San Diego) diluted 1:1000 and 1:500 in blocking buffer, respectively. ELISA and confocal microscopy were carried out as previously described (Bisht et al., 2004).

Immunization protocol and SARS-CoV challenge

Groups of seven female 6-week BALB/c mice were injected subcutaneously with 10 μg of nS protein or with an unrelated vaccinia virus protein L1R on days 0, 28, and 56. Approximately 4 weeks after the third immunization, mice were intranasally challenged with 10^5 $TCID_{50}$ of SARS-CoV in 50 μl. Two days later, their lungs and nasal turbinates were removed and SARS-CoV titers were determined as described (Subbarao et al., 2004). A non-parametric Mann–Whitney *U* test was used for statistical analysis.

Acknowledgments

The authors are grateful to Elaine Lamirande and Jadon Jackson, NIAID for their expert assistance.

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