Severe acute respiratory syndrome coronavirus spike protein expressed by attenuated vaccinia virus protectively immunizes mice

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The spike protein (S), a membrane component of severe acute respiratory syndrome coronavirus (SARS-CoV) is anticipated to be an important component of candidate vaccines. We constructed recombinant forms of the highly attenuated modified vaccinia virus Ankara (MVA) containing the gene encoding full-length SARS-CoV S with and without a C-terminal epitope tag called MVA/S-HA and MVA/S, respectively. Cells infected with MVA/S or MVA/S-HA synthesized a 200-kDa protein, which was recognized by antibody raised against a synthetic peptide of SARS-CoV S or the epitope tag in Western blot analyses. Further studies indicated that S was N-glycosylated and migrated in SDS polyacrylamide gels with an apparent mass of ∼160 kDa after treatment with peptide N-glycosidase F. The acquisition of resistance to endoglycosidase H indicated trafficking of S to the medial Golgi compartment, and confocal microscopy showed that S was transported to the cell surface. Intranasal or intramuscular inoculations of BALB/c mice with MVA/S produced serum antibodies that recognized the SARS S in ELISA and neutralized SARS-CoV in vitro. Moreover, MVA/S administered by either route elicited protective immunity, as shown by reduced titers of SARS-CoV in the upper and lower respiratory tracts of mice after challenge. Passive transfer of serum from mice immunized with MVA/S to naïve mice also reduced the replication of SARS-CoV in the respiratory tract after challenge, demonstrating a role for antibody to S in protection. The attenuated nature of MVA and the ability of MVA/S to induce neutralizing antibody that protects mice support further development of this candidate vaccine.

Severe acute respiratory syndrome (SARS), an emerging infectious disease of humans, appeared in China in November 2002 and spread to 30 countries in early 2003. Before the epidemic ended, 8,098 probable cases of SARS and 774 associated deaths were reported to the World Health Organization (www.cdc.gov/mmwr/mguide/sars.html). The etiologic agent of SARS infections is uncertain (7). Other members of the CoV family can cause fatal diseases of livestock, poultry, and laboratory rodents (8). The two previously identified human SARS infections are not severe (5, 6). Closely related CoVs were recovered from civet cats and other animals in southern China, although the source of human SARS-CoV S of the Urbani strain was identified as angiotensin-converting enzyme 2 (12, 13).

Although the 2002/2003 epidemic was eventually controlled by case isolation, the high morbidity and mortality, lack of effective treatment, and potential for reemergence make it imperative to develop effective means to prevent or cure the disease should it reappear. As an initial step, a rodent animal model was developed in which SARS-CoV replicates but does not cause disease (14). Importantly, prior infection or transfer of convalescent serum prevented replication of SARS-CoV in the respiratory tract of mice. The purpose of the present study was to determine whether expression of the S alone could raise neutralizing antibodies and protectively immunize mice.

Vaccinia virus vectors, including the highly attenuated modified vaccinia virus Ankara (MVA) strain, have been used to express and characterize glycoproteins of numerous pathogens, and some of those are being evaluated as candidate prophylactic and therapeutic vaccines (15). MVA accumulated multiple deletions and other mutations during >500 passages in chicken embryo fibroblasts (CEF) (16–18), resulting in a severe host range restriction in most mammalian cells (19–21). Because the restriction occurs at a late stage of virus assembly, MVA expresses viral and recombinant proteins in nonpermissive as well as in permissive cells (22). MVA is highly attenuated due to its replication defect in mammalian cells, and no adverse effects were reported even when high doses of MVA were given to immune deficient nonhuman primates (23) or severe combined immunodeficiency disease mice (24). Here, we show that the full-length S of SARS-CoV, expressed by MVA, induces binding and neutralizing antibody and protectively immunizes mice against a subsequent infection with SARS-CoV.

Materials and Methods

Viruses and Cells. Primary CEF cells prepared from 10-day-old embryos were grown in minimum essential medium supplemented with 10% FBS and was used to propagate and titer MVA and recombinant MVA strains.

Recombinant Virus Construction. The 3,768-nt ORF encoding the SARS-CoV S of the Urbani strain was copied and amplified from SARS-CoV virion RNA by RT-PCR, and was cloned and sequenced. A clone was identified that exactly matched the genome sequence established it as a new member of the family (5, 6). A 412-bp PCR fragment encoding the SARS-CoV S was cloned into MVA to construct MVA/S-HA and MVA/S, respectively. The 3,768-nt ORF encoding the SARS-CoV S is quite divergent from those of other CoVs, exhibiting only 20–27% overall amino acid identity (5). Recent studies indicated that the SARS-CoV S is expressed as a noncleaved glycoprotein with an apparent mass of 180–200 kDa that interacts with a functional receptor identified as angiotensin-converting enzyme 2 (12, 13).

Abbreviations: SARS, severe acute respiratory syndrome; CoV, coronavirus; S, spike protein; CEF, chicken embryo fibroblast; MVA, modified vaccinia virus Ankara; pdv, plaque forming unit; HA, hemagglutinin; endo, endoglycosidase; PNGase, peptide N-glycosidase; i.n., intranasal; TCID50, tissue culture 50% infective dose.

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tagenesis kit (Invitrogen). After mutagenesis, the entire S gene was PCR-amplified with or without an influenza virus hemagglutinin (HA) epitope tag and inserted into the XmaI site of the pLW44 transfer vector (provided by L. Wyatt, National Institutes of Health), bringing it under the control of the vaccinia virus modified H5 early late promoter (25) and adjacent to the gene encoding enhanced GFP regulated by the vaccinia virus P11 late promoter. The correct sequence of the entire S DNA insert was confirmed and recombinant MVAs were made by transfecting plasmids into CEF that were infected with 0.05 plaque forming units (pfu) of MVA per cell. Fiorescent plaques were cloned by six successive rounds of plaque isolation, propagated in CEF, and purified by sedimentation through a sucrose cushion (26). Titers of MVA/S and MVA/S-HA were determined by staining plaques with anti-vaccinia virus rabbit and anti-HA mouse antibodies, respectively.

Western Blotting. CEF and HeLa cells were infected with 5 pfu of recombinant MVA for 18 h. Infected cells were lysed in ice-cold RIPA buffer [50 mM Tris-HCl (pH 7.5)/150 mM NaCl/0.1% Triton X-100/0.1% SDS/0.5% sodium deoxycholate] supplemented with protease inhibitor mixture (Sigma). Lysates were kept on ice for 10 min, were centrifuged, and were resolved by SDS-PAGE of lysates of cells infected with MVA and MVA/S-HA and incubated for 1 h at room temperature with anti-HA mouse mAb (Covance Research Products, Berkeley, CA) or anti-SARS-CoV S rabbit polyclonal antibody (IMG-Science) diluted in blocking buffer, washed again and incubated with horseradish peroxidase-conjugated secondary anti-rabbit antibody (Roche Applied Science) diluted in blocking buffer, washed again and incubated with substrate solution (3,3′-5,5′-tetramethylbenzidine, Roche Applied Science). The difference in absorbance at 450 and 492 nm was determined, readings from wells lacking antigen were subtracted, and endpoint titers were calculated when the absorbance difference was <0.1.

Neutralization Assay. Neutralizing antibody was determined by the inhibition of cytopathic effects mediated by SARS-CoV on Vero cell monolayers as described (14). The dilution of serum that completely prevented cytopathic effect in 50% of the wells was calculated (27).

Animal Challenge Experiments. Groups of eight BALB/c mice were inoculated intranasally (i.n.) or i.m. with 10⁴ pfu of MVA or MVA/S at 0 and 4 weeks. Four weeks after the second immunization, animals were challenged i.n. with 10⁴ tissue culture 50% infective dose (TCID₅₀) of SARS-CoV as described (14). Two days later, the lungs and nasal turbinate of four animals in each group were removed and the SARS-CoV titers were determined (14).

To obtain serum for passive protection studies, two groups of eight BALB/c mice received MVA/S or MVA i.m. at 0 and 4 weeks. Three weeks after the last immunization, sera were collected and pooled. Undiluted or diluted MVA/S or MVA serum in a total volume of 0.4 ml was injected i.p. in two to four naive mice. Mice were bled the following day to determine their levels of SARS-CoV-specific neutralizing antibody, and then each was challenged with 10⁵ TCID₅₀ of SARS-CoV and analyzed as above.

Results

Characterization of SARS-CoV S Expressed by Recombinant MVA. A cDNA clone containing the entire ORF encoding SARS-CoV S was modified by introducing silent mutations that eliminated two poxvirus transcription termination signals and was placed under the control of a vaccinia virus early/late promoter (mHS) and inserted by homologous recombination into the site of an existing deletion (del III) within the MVA genome to produce MVA/S-HA, with a 9-aa HA epitope tag coding sequence inserted by homologous recombination into the site of an existing deletion (del III) within the MVA genome to facilitate the screening and isolation of recombinant viruses by repeated plaque purifications. Both viruses replicated well in CEF, and the SARS-CoV S insert was genetically stable, as assayed by plaque immunostaining with S-specific antibodies.

A protein doublet with an estimated mass of ~200 kDa, significantly higher than the value of 135 kDa for the unmodified protein predicted from the nucleotide sequence, was detected by SDS-PAGE of lysates of cells infected with MVA/S and MVA/S-HA and Western blotting with polyclonal antibody to S or a specific antibodies.

Confocal Microscopy. CEF or HeLa cells on coverslips were infected with 5 pfu per cell of MVA, MVA/S, or MVA/S-HA, incubated for 18 h, and were either left unfixed and unpermeabilized or fixed with cold 4% paraformaldehyde in PBS for 20 min at room temperature and permeabilized with 2.5% digitonin in PBS for 5 min on ice. The coverslips were washed and incubated with anti-SARS-CoV S-HA mouse serum kindly provided by L. Anderson (Centers for Disease Control, Atlanta) or anti-HA mouse mAb for 1 h at room temperature, washed again, and were incubated with Alexa 594-conjugated-anti-mouse IgG (Molecular Probes) diluted in PBS containing 10% FBS for 30 min at room temperature. Coverslips were mounted in 20% glycerol and examined with an inverted confocal microscope.

ELISA. A 96-well plate was coated overnight at 4°C with 50 ng per well of soluble recombinant protein containing the S1 domain of SARS-CoV S made in insect cells, blocked with 5% skimmed milk in PBS containing 0.2% Tween-20 for 1 h at 37°C, and incubated with two-fold dilutions of serum from immunized or immunized mice for 1 h at 37°C. After extensive washes, the plate was incubated for 1 h with horse radish peroxidase-conjugated secondary anti-mouse antibody (Roche Applied Science) diluted in blocking buffer, washed again, and incubated with substrate solution (3,3′-5,5′-tetramethylbenzidine, Roche Applied Science). The difference in absorbance at 450 and 492 nm was determined, readings from wells lacking antigen were subtracted, and endpoint titers were calculated when the absorbance difference was <0.1.

Characterization of SARS-CoV S Expressed by Recombinant MVA. A cDNA clone containing the entire ORF encoding SARS-CoV S was modified by introducing silent mutations that eliminated two poxvirus transcription termination signals and was placed under the control of a vaccinia virus early/late promoter (mHS) and inserted by homologous recombination into the site of an existing deletion (del III) within the MVA genome to produce MVA/S-HA, with a 9-aa HA epitope tag coding sequence inserted by homologous recombination into the site of an existing deletion (del III) within the MVA genome to facilitate the screening and isolation of recombinant viruses by repeated plaque purifications. Both viruses replicated well in CEF, and the SARS-CoV S insert was genetically stable, as assayed by plaque immunostaining with S-specific antibodies.

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The SARS-CoV S has 23 potential N-linked glycosylation sites (5), the use of which could contribute to the mass of the protein determined by SDS/PAGE. To evaluate this possibility, S expressed in HeLa cells was treated with PNGase F, which hydrolyzes all types of N-glycan chains. PNGase F treatment converted the 200-kDa doublet to a single sharp band of 160 kDa (Fig. 2A), which was still greater than the 135 kDa estimated from the gene sequence. However, differences of this magnitude between the theoretical mass and the mass estimated by SDS/PAGE are commonly found, and this discrepancy does not necessarily indicate that S contains additional posttranslational modifications.

Further experiments were carried out by using endo H, which digests the N-linked high-mannose carbohydrate side chains of glycoproteins that are synthesized in the endoplasmic reticulum, but not after conversion to a more complex form in the medial Golgi apparatus. Only a subpopulation of S was digested, because both the original size protein and a faster migrating one were detected (Fig. 2A). The latter had a slightly higher mass than the PNGase F-treated protein, which is consistent with N-acetylglucosamine residues remaining after hydrolysis by endo H. To determine the kinetics of acquisition of endo H resistance, cells infected with MVA/S-HA were pulse-labeled for 10 min with [35S]methionine and [35S]cysteine and were then chased in medium supplemented with unlabeled cysteine and methionine. Cells were lysed immediately after the pulse or chase and the S was captured with anti-HA affinity matrix, subjected to endo H digestion, resolved by SDS/PAGE, and visualized by autoradiography. The molecular masses of marker proteins in kDa are shown on the left.
Cellular Localization of S. The glycosylation and partial resistance to endo H was consistent with trafficking of the SARS-CoV S through the endoplasmic reticulum to the Golgi compartment. To determine whether S was expressed at the cell surface, unpermeabilized CEFs that had been infected with MVA/S were stained with antibody to S followed by Alexa 594-conjugated-anti-mouse IgG. Whereas the fluorescence due to coexpressed GFP was present throughout the cytoplasm, the labeling of S was restricted to the cell surface (Fig. 3D). Moreover, no labeling occurred in cells infected with the MVA vector (Fig. 3B) or uninfected cells (data not shown). Experiments were also carried out with cells infected with MVA/S-HA except that antibody to the epitope tag was used. The absence of staining of unpermeabilized cells (Fig. 3F) was consistent with the S protein having a type I topology with the tagged C terminus in the cytoplasm. After permeabilization of the plasma membrane with digitonin, both plasma membrane and juxtanuclear staining were evident (Fig. 3H). Similar patterns were found when infected HeLa cells were examined by confocal microscopy (data not shown).

Immunogenicity of MVA/S in Mice. Mice were inoculated i.n. or i.m. with $10^2$ pfu of MVA/S at time 0 and again at 4 weeks. Antibody was determined by an endpoint ELISA using a recombinant protein consisting of the S1 domain of SARS-CoV made in insect cells and purified by affinity chromatography. Antibody was detected at 4 weeks and peaked at 6 weeks (Fig. 4A). Similar titers were obtained after either route of inoculation. The titers began to decline with time and were not boosted at 2 weeks after the SARS-CoV challenge described in the next section (Fig. 4A).

The ability of sera to neutralize SARS-CoV infectivity for VERO cells was determined as described (14). Neutralizing antibody was detected after the second immunization by either the i.n. or i.m. route (Fig. 4B).

Protection of Mice Immunized with MVA/S. Previous studies (14) demonstrated that mice inoculated i.n. with SARS-CoV exhibit no overt signs of disease, but have elevated virus titers in the respiratory tract that peak within 2 days and are cleared by 7 days. For the present study, we had three control and two experimental groups. The controls were mice that were uninoculated or that had received the MVA vector i.m. or i.n. When these mice were challenged with $10^4$ TCID$_{50}$ of SARS-CoV, $\approx 10^5$ TCID$_{50}$ of SARS-CoV per g of lung was recovered on day 2 (Fig. 5). By contrast, the titers of SARS-CoV from the lungs of mice immunized with MVA/S either i.m. or i.n. were reduced to levels that were barely above the limit of detection (Fig. 5). Approximately $10^4$ TCID$_{50}$ per g of SARS-CoV were recovered from the nasal turbinates of control mice, but this amount too was significantly reduced in the immunized animals (Fig. 5). The severe reduction in SARS-CoV replication may explain the absence of an anamnestic ELISA antibody response to S after challenge (Fig. 4A). Neutralizing titers to SARS-CoV were not measured after challenge.

Passive Protection Mediated by Serum from MVA/S-Immunized Mice. MVA can induce both humoral and cell mediated immune responses. To determine a role for antibody, we pooled sera from mice that had been immunized i.m. with $10^2$ pfu of MVA/S or MVA on day 0 and 28 and were bled 3 weeks later. The reciprocal ELISA titer to S1 was $\approx 1:25,000$ and the mean neutralizing titer was 1:284. Undiluted or diluted serum (0.4 ml) was administered i.p. to naive mice to evaluate the protective role of antibody. As a positive control, we administered hyperimmune SARS-CoV serum to two mice (14). On the next day, the mice received an i.n. challenge of $10^6$ TCID$_{50}$ of SARS-CoV, and 2 days later, their nasal turbinates and lungs were removed to measure the virus titers. Administration of undiluted MVA/S serum reduced the lung titers by $10^{3.1}$ (Table 1), compared with recipients of MVA control serum, indicating that antibodies to the SARS-CoV S conferred the observed protection. Protection was observed despite only achieving a neutralization titer of 1:35 in recipient mice. Replication of SARS-CoV increased as the quantity of passively transferred serum decreased, but significant reductions in lung virus titers still occurred at dilutions of 1:4, 1:16, and 1:64. The absence of detectable neutralizing antibody in mice receiving these dilutions of passively transferred serum probably reflects a low sensitivity of the in vitro neutralization assay.
Our object was to express S in a native state to induce antibodies that would neutralize SARS-CoV. The secretory pathway of the cell has an important quality control function, and the trafficking of a protein from the endoplasmic reticulum to the plasma membrane is a sign of proper folding. The N-linked oligosaccharide pathway is frequently used for tracking protein movement. Addition of N-linked oligosaccharides occurs in the endoplasmic reticulum and the conversion of the high mannose form to complex endo H-resistant N-linked chains occurs on transport from the cis to the medial Golgi compartment. We found that the S ORF of SARS-CoV was expressed by recombinant MVA as a protein of ~200 kDa, which was reduced to 160 kDa by a glycosidase specific for N-linked carbohydrates. Trafficking of S to the medial Golgi apparatus was indicated by acquisition of endo H resistance by a subpopulation of molecules within 40 min after pulse labeling. The staining of the surface of unpermeabilized cells infected with MVA/S by S-specific antibody provided direct evidence for insertion into the plasma membrane. Furthermore, the inability of antibody to a C-terminal epitope tag to stain cells unless they were permeabilized indicated that S has a type 1 topology in the membrane. We did not detect putative S1 and S2 cleavage products, as found for group 2 but not group 1 CoV S proteins (28). Xiao et al. (13) expressed full-length S by transfection and detected low amounts of several smaller than full-length S fragments, which they suggested might include specific cleavage products, although no evidence to support this hypothesis was presented. Our characterization studies strongly suggested that MVA expressed a properly folded form of S, leading us to determine whether it would elicit neutralizing antibodies.

Mice immunized with MVA/S by i.n. or i.m. routes developed antibodies that bound to the S1 domain of S and neutralized SARS-CoV in vitro. Furthermore, mice immunized i.m. or i.n. exhibited little or no replication of SARS-CoV in the upper and lower respiratory tracts after an i.n. inoculation. Control mice vaccinated with the MVA vector by i.n. or i.m. routes were unprotected, indicating that the effect was specific for the expressed S protein and was not due to enhanced nonspecific immunity. The ability of i.m. or i.n. inoculation of a recombinant MVA to prevent upper and lower respiratory infections had previously been found in a rodent model of parainfluenza virus 3 (25).

Discussion

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Previous studies (14) showed that i.p. inoculation of hyperimmune serum from mice inoculated twice with SARS CoV provided protection against SARS-CoV in the lower respiratory tract and to a lesser extent in the upper respiratory tract (14). Protection with serum from MVA/S-immunized animals was demonstrated in the present study. Because serum from animals inoculated with the MVA vector had no effect, we can attribute the protection to S-specific antibodies. These results indicated that the S of SARS CoV, like that of other CoV, is an important target of neutralizing antibody both in vitro and in vivo. Recently, mAbs specific to SARS-CoV S1 domain with in vitro neutralizing
activity were described (29), and it will be interesting to test them in vivo.

No enhanced virus replication or obvious disease was found in mice that were immunized with MVA/S before challenge with SARS-CoV, as has been found after immunization with a vaccinia virus vector expressing S from feline infectious perminitis virus and challenge with the corresponding virus (30). The latter effect is thought to be due to S antibody-dependent enhanced infection of macrophages (31, 32). Although it will be necessary to look for enhanced antibody effects in other animal models of SARS-CoV, the present study is encouraging for the development of SARS-CoV vaccines based on the highly attenuated MVA vector expressing S.

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