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Middle East Respiratory Syndrome Coronavirus Spike Protein Delivered by Modified Vaccinia Virus Ankara Efficiently Induces Virus-Neutralizing Antibodies

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Middle East respiratory syndrome coronavirus (MERS-CoV) has recently emerged as a causative agent of severe respiratory disease in humans. Here, we constructed recombinant modified vaccinia virus Ankara (MVA) expressing full-length MERS-CoV spike (S) protein (MVA-MERS-S). The genetic stability and growth characteristics of MVA-MERS-S make it a suitable candidate vaccine for clinical testing. Vaccinated mice produced high levels of serum antibodies neutralizing MERS-CoV. Thus, MVA-MERS-S may serve for further development of an emergency vaccine against MERS-CoV.

MERS-CoV infection remains unclear. The virus is suspected to persist in animal reservoirs and cause zoonotic infections in humans (4, 5). The MERS-CoV spike (S) protein, a characteristic structural component of the virion membrane, forms large protruding spikes on the surface of the virus; its S1 domain mediates binding to dipeptidyl peptidase 4, which serves as the host cell receptor of MERS-CoV (6). Importantly, the S protein is considered a key component of vaccines against coronavirus infection, including severe acute respiratory syndrome (SARS) (7, 8).

Modified vaccinia virus Ankara (MVA), a highly attenuated strain of vaccinia virus originating from growth selection on chicken embryo fibroblasts (CEF), shows a characteristic replication defect in mammalian cells (9, 10, 11). At present, MVA serves as one of the most advanced recombinant poxvirus vectors in preclinical research and human clinical trials for developing new vaccines against infectious disease and cancer (12, 13, 14).

Here, we show that the full-length S protein of MERS-CoV, expressed by MVA, is produced as an ~210-kDa N-glycosylated protein that is specifically recognized by antibodies in Western blot analysis. Further studies suggest cleavage of the mature full-length S glycoprotein into an amino-terminal domain (S1) and an ~85-kDa carboxy-terminal domain (S2) that is putatively anchored to the membrane. When tested as a vaccine in mice, recombinant MVA expressing the S protein induced high levels of circulating antibodies that neutralize MERS-CoV in tissue culture infections.

Construction and characterization of recombinant MVA. cDNA containing the entire gene sequence encoding MERS-CoV S (GenBank accession no. JX869059) was obtained by DNA synthesis (Invitrogen Life Technology, Regensburg, Germany) and modified by introducing silent mutations that remove three termination signals (TTTTTNT) for vaccinia virus transcription (MERS-S). Furthermore, we generated a second version containing a tag sequence encoding nine amino acids (YPYDVPDYA) from influenza virus hemagglutinin (HA tag) attached at the C terminus of S (MERS-SHA). MERS-S and MERS-SHA were cloned under the transcriptional control of the vaccinia virus early/late promoter PmH5 (15) and introduced by homologous recombination into an existing deletion site (deletion III) in the MVA genome (Fig. 1A).

MVA expressing MERS-S or MERS-SHA (MVA-MERS-S or MVA-MERS-SHA respectively) was obtained using standard methods to generate recombinant MVA vaccines suitable for clinical testing, as described previously (13). Briefly, transient coproduction of the fluorescent marker protein mCherry (under the control of the vaccinia virus late promoter P11 [16]) was used to isolate clonal recombinant viruses by screening for fluorescent cell foci during repetitive plaque purification. At this stage, immunostaining of infected cell cultures with anti-HA tag monoclonal or polyclonal antibodies from MERS-CoV-infected macaques suggested synthesis of the recombinant SHA and S proteins in CEF and Vero cells (ATCC CCL-81) (Fig. 2). MVA-MERS-S and MVA-MERS-SHA were genetically stable and replicated efficiently in CEF but not in human HeLa or HaCat cells (Fig. 1B and C). The latter findings confirmed that the recombinant viruses could be handled under biosafety level 1 conditions.

Characterization of MERS-CoV S produced by recombinant MVA. We specifically detected a protein with an estimated molecular mass of about 200 kDa in lysates from MVA-MERS-S- and MVA-MERS-SHA-infected Vero cells by using sera from MERS-
CoV-infected macaques in Western blots (Fig. 3A, upper panel). Further immunoblot analysis with monoclonal anti-HA tag antibody (Roche, Penzberg, Germany) confirmed the production of a protein doublet of 200 to 210 kDa in cells infected with MVA-MERS-SHA as well as a second protein of about 85 kDa (Fig. 3A, lower panel).

The molecular masses of the 200- to 210-kDa polypeptides detected by SDS-PAGE were significantly higher than the 149 kDa predicted for MERS-CoV S protein based on its nucleotide sequence. NetNGlyc server analysis suggested at least 17 likely N-glycosylation sites [Asn-X-(Ser/Thr)] for co- and posttranslational modification. Therefore, we investigated the glycosylation pattern of the expressed SHA protein by using treatment with peptide-N-glycosidase F (PNGase F) or endoglycosidase H (endo H) (both from New England BioLabs), followed by Western blotting (Fig. 3B). SHA produced in MVA-MERS-SHA-infected Vero cells was treated with PNGase F, which removes all N-linked oligosaccharide chains from glycoproteins. This treatment reduced the ∼210-kDa protein doublet to a sharp protein band of 150 kDa, a value closely matching the predicted mass of unmodified MERS-CoV S protein.

Similarly, PNGase F treatment converted the 85-kDa protein fragment to a polypeptide with a molecular mass of 55 kDa. Endo H cleaves N-linked high-mannose oligosaccharides synthesized
in the endoplasmic reticulum but not those matured to more complex oligosaccharides in the Golgi apparatus. Western blot analysis of SHA digested with endo H revealed a band of 150-kDa proteins, indicating complete hydrolysis of N-linked oligosaccharides. An additional subpopulation of SHA migrated with the original size of about 210 kDa, indicating resistance to endo H treatment. Of note, the 85-kDa protein subpopulation remained largely unaffected by endo H digestion.

**Immunogenicity of MVA-MERS-S in mice.** BALB/c mice were vaccinated intramuscularly with 10⁶ PFU MVA-MERS-S at 0 and 3 weeks. Twenty days after the first immunization and 10 days after the second immunization, serum samples were tested for their capacity to neutralize MERS-CoV (EMC isolate) in tissue culture infections by using 200 50% tissue culture infective doses (TCID₅₀) in Vero cells or 100 TCID₅₀ in Huh-7 cells. Even a single application of MVA-MERS-S induced low levels of virus-neutralizing antibodies in all eight animals tested (Fig. 4A). After booster immunization, all vaccinated animals produced high levels of circulating antibodies that neutralized MERS-CoV (Fig. 4B and C). In contrast, neutralizing antibodies were not detected in serum samples from control animals inoculated with nonrecombinant MVA or saline (phosphate-buffered saline [PBS]). The specificity of the induced MVA-MERS-S antibodies for MERS-CoV was confirmed by the absence of detectable neutralization against SARS coronavirus (SARS-CoV) (strain HKU39849) (Fig. 4D). In addition, the MERS-CoV-neutralizing activity of these serum samples could be only partly blocked by preincubation with proteins that encompass the receptor binding domain (RBD) (Fig. 4E and F).

This last observation is consistent with the fact that besides the RBD, other parts of the spike protein are able to induce neutralizing antibodies (17).

**Conclusions.** Our objective was to use MVA vectors compatible with clinical evaluation to express mostly native S antigen to induce antibodies that would neutralize MERS-CoV. The S gene of MERS-CoV expressed by recombinant MVA produced a glycoprotein migrating at a molecular mass of about 210 kDa. Glycosidase treatment to remove all N-linked carbohydrates resulted in a polypeptide of 150 kDa, closely corresponding to the molecular mass predicted from the S gene nucleotide sequence. A subpopulation of protein apparently acquired endo H resistance, indicating trafficking of S to the Golgi apparatus, which correlates with observations made for SARS-CoV S protein (18,19, 20).

In addition, we obtained evidence for putative S1 and S2 cleavage of full-length S as predicted recently (6, 21). The HA tag fused to the C terminus of S₁₂₅₉ enabled us to detect two glycoprotein subpopulations, full-length S (~210 kDa) and subdomain S2 (~85 kDa), by anti-HA Western blot analysis (Fig. 3). We considered furin cleavage sites because spike proteins of some betacoronaviruses and all gammacoronaviruses are typically activated by intracellular furin-dependent cleavage (22, 23; for a review, see...
ACKNOWLEDGMENTS

We thank Berend Jan Bosch for providing recombinant MERS-CoV receptor binding domain protein and sera from rabbits immunized with this protein.

F.S. is a grantee of China Scholarship Council (CSC) and LMU Munich. This study was partly supported by the German Centre for Infection Research (DZIF; TTU 01.901), and the European Union FP7 projects EMPERIE (contract no. 223498) and ANTIGONE (contract no. 278976).

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