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Recombinant Modified Vaccinia Virus Ankara Expressing the Spike Glycoprotein of Severe Acute Respiratory Syndrome Coronavirus Induces Protective Neutralizing Antibodies Primarily Targeting the Receptor Binding Region

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Immunization with a killed or inactivated viral vaccine provides significant protection in animals against challenge with certain corresponding pathogenic coronaviruses (CoVs). However, the promise of this approach in humans is hampered by serious concerns over the risk of leaking live severe acute respiratory syndrome (SARS) viruses. In this study, we generated a SARS vaccine candidate by using the live-attenuated modified vaccinia virus Ankara (MVA) as a vector. The full-length SARS-CoV envelope Spike (S) glycoprotein gene was introduced into the deletion III region of the MVA genome. The newly generated recombinant MVA, ADS-MVA, is replication incompetent in mammalian cells and highly immunogenic in terms of inducing potent neutralizing antibodies in mice, rabbits, and monkeys. After two intramuscular vaccinations with ADS-MVA alone, the 50% inhibitory concentration in serum was achieved with reciprocal sera dilutions of more than 1,000- to 10,000-fold in these animals. Using fragmented S genes as immunogens, we also mapped a neutralizing epitope in the region of N-terminal 400 to 600 amino acids of the S glycoprotein (S400-600), which overlaps with the angiotensin-converting enzyme 2 (ACE2) receptor-binding region (RBR; S318-510). Moreover, using a recombinant soluble RBR-Fc protein, we were able to absorb and remove the majority of the neutralizing antibodies despite observing that the full S protein tends to induce a broader spectrum of neutralizing activities in comparison with fragmented S proteins. Our data suggest that a major mechanism for neutralizing SARS-CoV likely occurs through blocking the interaction between virus and the cellular receptor ACE2. In addition, ADS-MVA induced potent immune responses which very likely protected Chinese rhesus monkeys from pathogenic SARS-CoV challenge.

First evident in Guangdong province of China around November 2002, severe acute respiratory syndrome (SARS) emerged as a human infectious disease caused by a novel variant of coronavirus (SARS-associated coronavirus, SARS-CoV) (12, 13, 20, 21, 32). From April to June of 2003, SARS-CoV quickly became a global outbreak, causing tremendous public panic. SARS is characterized by severe pulmonary infection with a high degree of transmissibility and mortality (8, 22, 34). The modes of SARS-CoV transmission include shedding of the virus from respiratory tract via droplets, close contact, and fomites (56). Once people become infected, they develop influenza-like symptoms: high-grade fever and chills, myalgia, headache, cough with or without sputum production, loss of appetite, and dyspnea (5, 19). Chest radiographic examinations reveal unilateral or bilateral areas of consolidation, predominantly around the periphery (5, 28–31). The incubation period of SARS ranges from 2 to 16 days, with a mean incubation of 6.4 days (7). The estimated case mortality rates are 13.2% for patients younger than 60 years and 43.3% for patients aged 60 years or older (10). The outbreak of SARS-CoV affected 33 countries and regions in the world across five continents. By the end of June 2003, there were 8,450 cases and 810 deaths (www.cdc.gov/mmwr/mguide_sars.html).

Although the SARS outbreak has subsided since the summer of 2003, sporadic cases emerged within the Southern part of China recently. These cases were subsequently traced back to an animal origin, which provided further evidence that SARS is likely a result of zoonotic infection in humans (15, 16, 27, 36, 37). To this end, an unknown animal reservoir provides additional difficulties on the prevention of the virus. Worst of all, the accidental infection of two laboratory workers recently caused a small outbreak in China, suggesting the need for more stringent virus containment (14, 25, 27). Up to now, there are still no reliable diagnostic tests in the early stages of the disease, no effective antiviral therapy, and no preventive vaccines for human or animal use, which remain to be the three major challenges to fight the SARS epidemic in the future.

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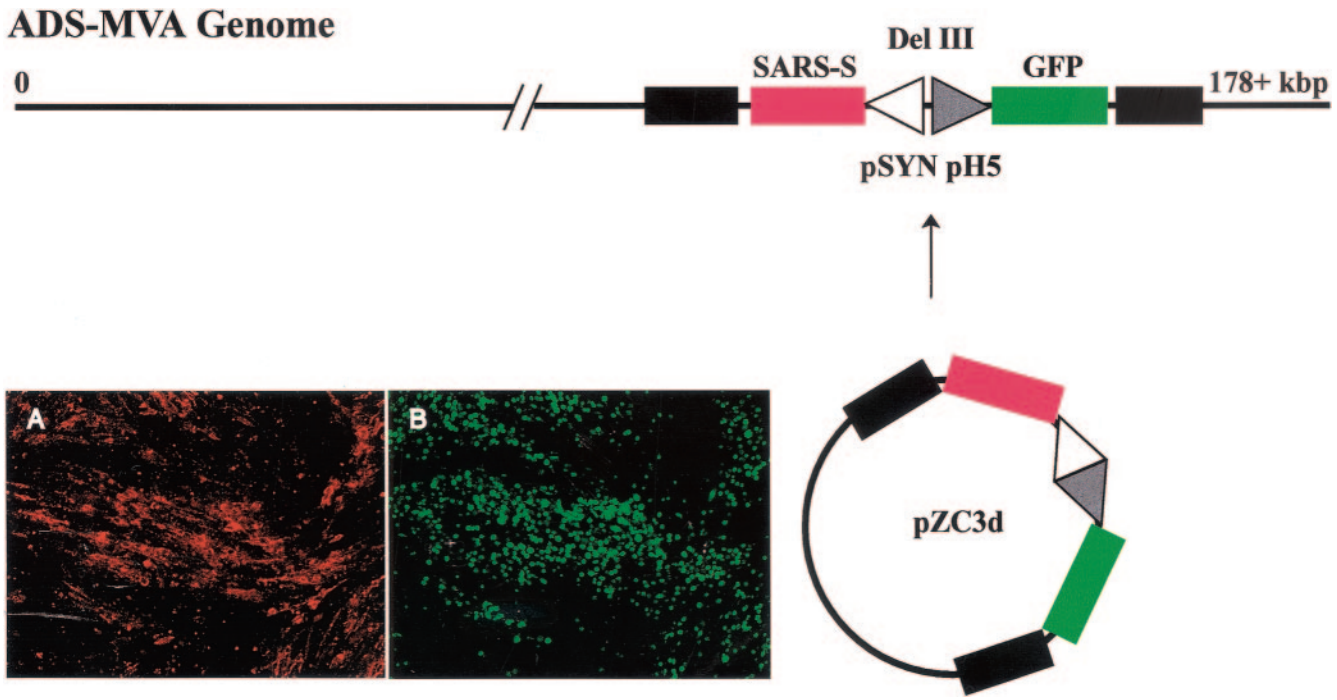

FIG. 1. Schematic representation of ADS-MVA construction (top and right panels) and the characterization of ADS-MVA by using avian cells (left bottom panel). The SARS-S gene was introduced, together with GFP gene, each under a separate promoter, into the Del III region of the MVA genome. (A) S glycoprotein was detected on CEF cells by using an anti-S polyclonal antibody (WH) in an immunofluorescence assay. (B) GFP coexpressed on the same cell population as seen in panel A.

Therefore, the rapid development of a safe and effective vaccine remains to be one of the highest priorities in fighting SARS.

Recently, Li et al. identified angiotensin-converting enzyme 2 (ACE2) as a functional receptor for SARS-CoV (23). This finding was confirmed by other research groups (47, 54). The interaction between ACE2 and SARS-CoV S glycoprotein was further explored to elucidate the structure and function of ACE2 and of the viral envelope protein (33). ACE2 was found to interact with an independently folded receptor-binding domain, a 193-residue fragment (S318-510), of the SARS-CoV S protein (50). This 193-residue fragment alone also exhibited potent activity to block S-protein-mediated infection. This finding suggested that this fragment contains the necessary functional element to compete with the live virus for the binding to ACE2. In a separate study, a human monoclonal antibody (scFv 80R) specific for receptor-binding domain efficiently neutralized SARS-CoV and inhibited syncytium formation between cells expressing the S protein and those expressing ACE2 (42). These findings formed a firm basis for developing and studying the mechanism of vaccine induced neutralization targeted on the receptor-binding domain of S glycoprotein.

We used modified vaccinia virus Ankara (MVA) as a vector for SARS vaccine construction. We chose MVA because it is a host-range-restricted vaccinia virus strain. Unlike conventional vaccinia vaccinations which may cause nosocomial infection and even death in humans, MVA is unable to multiply in human and most other mammalian cells (11, 51) due to the loss of important host range genes in the viral genome (1, 2, 26). Nevertheless, the expression of genes in MVA DNA appears to be unaffected, since both early and late viral proteins are synthesized in human cells (43, 44). More importantly, MVA has been used in large vaccine trials and in clinical practice for primary vaccination of more than 100,000 humans against smallpox (18, 38). No side effects have been associated with its use, even when high-risk patients or experimentally immunesuppressed monkeys received primary vaccination (39–41).

In the present study, the full-length SARS-CoV envelope S-glycoprotein gene was introduced into the deletion III region of the MVA genome. We chose S glycoprotein because it mediates the initial stages of viral entry and serves as the major targets recognized by both humoral and cellular immune responses (23). The newly generated recombinant ADS-MVA was able to induce high levels of neutralizing antibodies in mice, rabbits, and monkeys after two intramuscular vaccinations. The neutralizing antibodies induced in Chinese rhesus monkeys very likely protected the animals from pathogenic SARS-CoV challenge. Our data demonstrate that ADS-MVA may serve as a potent and safe prophylactic vaccine candidate against SARS-CoV infection in both human and animals. In addition, a major neutralizing epitope has been mapped to a region within the ACE2-binding region of the viral S glycoprotein which may have critical implications for vaccine development.

MATERIALS AND METHODS

MVA and insertion vectors. The original MVA virus and the insertion vector pLW7 were kindly provided by Bernie Moss and Lynda Wyatt at the National Institutes of Health. Based on pLW7, we have constructed a dual-promoter

insertion vector, pZC3d, by introducing a synthetic pH5 promoter into the vector (Fig. 1). Both promoters are vaccinia virus-specific early/later promoters. The new vector allows us to incorporate multiple genes into a single recombinant MVA virus. Like pLW7, pZC3d still targets the deletion III region of the MVA genome.

Construction and purification of ADS-MVA. Using pZC3d, we were able to recombine the wild-type SARS full-length S gene and a reporter green fluorescent protein (GFP) gene into the deletion III region of MVA to generate ADS-MVA (Fig. 1). The S gene was originally obtained from SARS-CoV HKU39849, an isolate from Hong Kong (AY278491) (57). The recombinant ADS-MVA was generated by the homologous recombination method in chicken embryo fibroblast (CEF) cells (45, 52, 53). The positive cell focus was subsequently selected by using GFP under fluorescence microscope. Eight rounds of the focus purification were applied to purify the virus. For comparison purpose, we generated another recombinant virus, ADC-MVA, by introducing a modified HCV E1E2 gene into the same site.

Immunofluorescence assay. An immunofluorescence assay was developed for the detection of SARS-CoV S glycoprotein expressed on cell surface. Briefly, CEF cells were plated into six-well plates (2×10^6 cells per well) on day one. The plates were pretreated with concanavalin A (100 μg/ml) for 30 min and rinsed twice with phosphate-buffered saline (PBS) before use. The following day, the cells were infected with recombinant, serially diluted ADS-MVA (1:10). Two hours later, the culture medium was replaced with Dulbecco modified Eagle medium supplemented with 2% fetal calf serum and incubated at 37°C with 5% CO_2 for 48 h. The infected cells were then incubated with heat-inactivated patient convalescent serum (WH; 1:500) for 1 h, followed by incubation with Alexa Fluor 594-labeled goat anti-human immunoglobulin G (IgG; H+L; Molecular Probes) for 30 min. The cells were washed three times with PBS. Positive foci were identified by fluorescence microscopy. CEF cells infected with a control ADC-MVA were included as background staining for each experiment.

Preparation of ADS-MVA stocks. The virus stock was prepared and purified by using CEF cells according to a procedure described previously (35, 45, 52, 53). The purified virus was expanded in CEF cells by using a roller culture technique.

Western blot analysis. For transient expression experiments, 293T cells were transfected with mammalian expression vectors: pcDNA-S200, pcDNA-S400, pcDNA-S600, pcDNA-S800, pcDNA-Sopt9, or pcDNA3.1 control. After 48 h, the transfected cells (10^6) were washed in PBS and lysed on ice for 30 min in 200 μl of cell lysis buffer (50 mM Tris-HCl [pH 8.0], 137 mM NaCl, 2 mM EDTA, 0.5% NP-40, 10% glycerol, and 1 μg each of pepstatin, leupeptin, and pefabloc [Sigma]/ml). For some experiments, cell lysates made from vaccinia virus infected cells were also used in this test. The cell debris was removed after centrifugation at 14,000 rpm for 10 min at 4°C. Cleared cell lysates were subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. The separated proteins were then transferred onto a nitrocellulose membrane and subjected to antibody blotting. Positive serum samples were collected from rabbits immunized with pcDNA-S400 encoding the N-terminal 400 amino acids of the S protein by using an in vivo electroporation technique. Negative sera were derived from healthy animals received placebo injections.

Immunization of animals. Six- to eight-week-old female BALB/c mice were immunized by intramuscular (i.m.) injection of ADMVA at weeks 0 and 3. Different groups were inoculated with 5×10^6 50% tissue culture infective dose(s) ($TCID_{50}$) or 5×10^7 $TCID_{50}$ of ADS-MVA, or ADC-MVA, or a saline control. Two weeks after the second injection, the mice were sacrificed, and their blood samples were used for analysis. Two rabbits were immunized with 10^8 $TCID_{50}$ of ADS-MVA, and another two were immunized with 10^8 $TCID_{50}$ of ADC-MVA on days 0 and 28. Moreover, eight rhesus monkeys were immunized twice on days 0 and 28. Four of them received ADS-MVA, whereas the other four received ADC-MVA. The first dose for the immunization was 10^8 $TCID_{50}$, and the second dose was 3×10^8 $TCID_{50}$. Two weeks after each immunization, blood samples were collected and subjected to analysis.

For DNA vaccination, two rabbits in each group were immunized twice on days 0 and 28 with each naked 400-μg DNA plasmid (pcDNA-S200, pcDNA-S400, pcDNA-S600, and pcDNA-S800) by using an in vivo electroporation technique. Moreover, at 2 months after the last DNA vaccination, these rabbits were boosted twice with 10^8 $TCID_{50}$ of ADS-MVA with a 4-week interval between injections. Control rabbits were injected with similar DNA plasmids but expressing HCV E1E2 gene. These animals were also boosted with ADC-MVA in the same way. In each case, blood samples were collected and subjected to analysis 2 weeks after each immunization.

For the monkey challenge study, eight Chinese rhesus monkeys were immunized twice on days 0 and 28. Four of them received ADS-MVA, whereas the other four received ADC-MVA. The dose for both immunizations was 5×10^8 $TCID_{50}$. Half of the vaccine was given via the i.m. route, whereas the other half was delivered intranasally (i.n.). At 4 weeks after the second immunization, each of the animals was challenged with 10^5 $TCID_{50}$ of the pathogenic $SARS\text{-}CoV_{PUMC01}$ via the same i.n. route. Of note, the vaccine strain $SARS\text{-}CoV_{HKU39849}$ shares 100% sequence homology with the challenge strain $SARS\text{-}CoV_{PUMC01}$ in the S gene (AY350750). This experiment was performed in a P3 animal facility at the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences. All animal experiments were approved by the Animal Care and Use Committees.

Neutralization assay. A neutralization assay was established to determine the humoral immune responses generated by the vaccine. The neutralizing activity of heat-inactivated animal sera was determined by using a pseudotype viral entry assay. The assay was established according to a method that has been previously described (9). The pseudotype virus was generated by cotransfecting 293T cells with two plasmids pcDNA-Sopt9 and $pNL4\text{-}3Luc^+Env^-Vpr^-$ carrying the optimized S gene and a human immunodeficiency virus type 1 backbone, respectively. The serial diluted serum samples were incubated with equal amounts of pseudotype virus at 37°C for 1 h. The serum-virus mixtures were subsequently added into preseeded HEK 293T-ACE2 cells. After 56 h, the infected cells were lysed for the measure of luciferase activity.

Antibody absorption assay. A soluble recombinant ACE2 receptor-binding region (S310-510) fused with human Fc (RBR-Fc) was used to absorb the antibodies that specifically bind to the receptor-binding region. Specific anti-human IgG Fc-agarose (Sigma) was then used to remove RBR-Fc–antibody complexes. Briefly, the diluted sera were incubated with soluble RBR-Fc at room temperature for 45 min. Then, 10 μl of prewashed anti-human IgG–Fc-agarose was added to each well. This absorption step was performed on a microplate shaker at room temperature for about 2 h to prevent the precipitation of the carrier agarose. The microplates were centrifuged for 10 min at room temperature at 3,000 rpm for 5 min. The remaining unbound sera were subjected to the neutralization assay using the pseudotype virus. To ensure the specificity of the assay, a set of controls were carried out in parallel for comparison including anti-human IgG agarose alone and RBR-Fc alone.

In another experiment, since monkey sera contain antibodies cross-react with anti-human Fc antibodies coated on the carrier agarose beads, we had to take a different approach. We conjugated Sepharose 4 Fast-Flow beads (Amersham Biosciences) directly with RBR-Fc by using a chemical method as described by the manufacturer. Instead of using soluble RBR-Fc to absorb antibodies, the RBR-Fc-conjugated beads were used to absorb and remove monkey antibodies that bound to the RBR portion. The rest experiment procedure was as described above.

SARS-CoV isolation. Eight Chinese macaques were sacrificed on day 7 after virus challenge, and lung homogenates of each animal were generated and subjected to virus isolation. The virus isolation was performed by inoculating lung homogenates into Vero cells. After 1 h of absorption, the inocula were washed away and replaced with fresh culture medium. The cultures were kept for observing cytopathic effect(s) (CPE) for 10 days. Thereafter, if no CPE was found, the cultures were blindly used to inoculate a second set of Vero cells for another 10 day. However, if CPE was found, an immunofluorescence assay (see above) was performed to detect SARS-CoV specific antigen in Vero cells to avoid the false readout based on CPE.

RT-PCR. To detect the viral RNA in specimens of SARS-CoV-inoculated monkeys, the total RNA was extracted from various specimens by using MagNA Pure LC total nucleic acid isolation kit (Roche Diagnostics). The extracted RNA was then tested by using a commercial SARS-CoV reverse transcription-PCR (RT-PCR) kit (Roche Diagnostics).

RESULTS

Construction and in vitro characterization of the ADS-MVA. To properly incorporate the full-length SARS-CoV S glycoprotein gene into the MVA genome, we constructed a dual promoter insertion shuttle vector. Within this vector, the S gene was constructed under the strong synthetic promoter pSYN, whereas a reporter GFP gene was under a separate relatively weaker promoter pH5 (Fig. 1). Since both genes were included within the same insertion frame, the GFP served as a surrogate marker for the selection of recombinant MVA carrying the S gene. Using this technique, we were able to generate and to purify the recombinant virus ADS-MVA. We found that the S protein and GFP were coexpressed in the

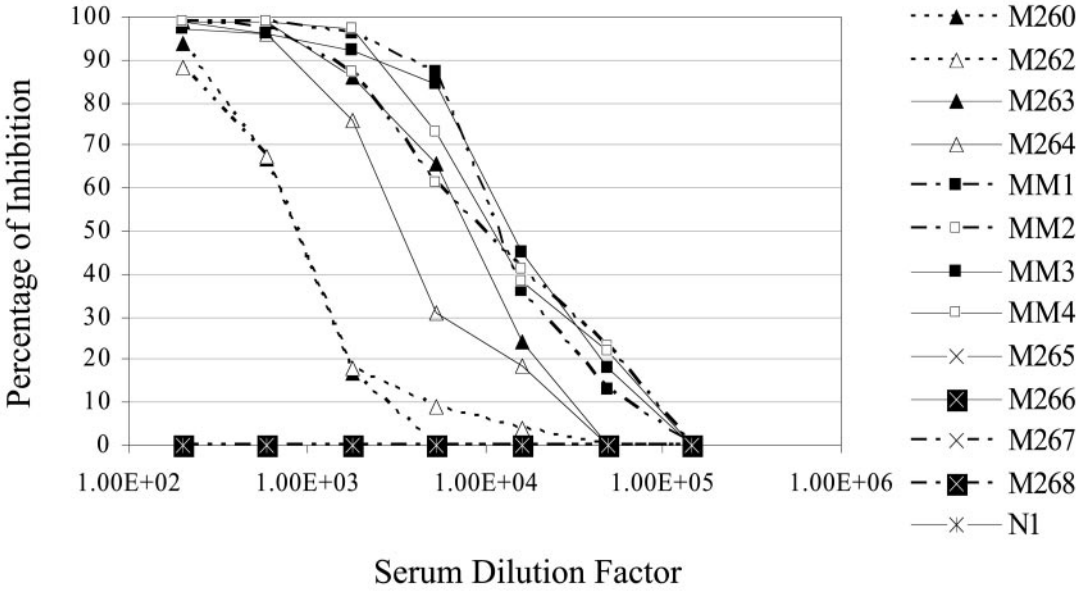

FIG. 2. Anti-SARS-CoV-specific neutralization antibody response in BALB/c mice after ADS-MVA vaccinations. Each mouse was designated with a number such as M260. The animals were immunized i.m. twice, 3 weeks apart, with 2×10^6 $TCID_{50}$ (M260 and M262) or 2×10^7 $TCID_{50}$ of ADS-MVA per mouse (M263, M264, and MM1 to MM4) or 2×10^7 $TCID_{50}$ of ADC-MVA (M265 to M268). Two control mice (N1 and N2) only received saline under the same immunization schedule.

same foci of ADS-MVA-infected CEF cells (Fig. 1A and B). The S protein was also detected by a rabbit antibody bound to the N-terminal 400 amino acids of the protein in a Western blot analysis. The S protein appeared at the position between molecular mass markers 160 and 250 kDa, which is bigger than the actual size predicted (data not shown). This migration pattern is probably due to the posttranslational modification of the protein (4, 54).

Considering that the introduction of the S gene might have altered the cell tropism of ADS-MVA, which may affect the safety profile of the vaccine, we tested the growth property of ADS-MVA in mammalian cells. We used ADS-MVA to infect several mammalian cells: 293 cells, HeLa cells, and Vero cells (data not shown). In contrast to the growing "patching" pattern overtime that is typically observed in permissive CEF cells (Fig. 1A), ADS-MVA infection in mammalian cells remained restricted in individually infected cells. Therefore, like the wild-type MVA, ADS-MVA did not productively replicate in mammalian cells, although the S glycoprotein was expressed. In addition, ADS-MVA was passaged on CEF cells nine times. These passages, however, did not lead to the loss of S expression, indicating that ADS-MVA is genetically stable (data not shown).

Immunogenicity of ADS-MVA in mice and rabbits. A pseudotype-based neutralization assay was established to characterize the immune sera generated in animals. The advantage of this assay is the elimination of using live SARS-CoV in the traditional neutralization assay. We used this assay to measure the serum neutralizing activity in animals immunized with ADS-MVA. First, eight mice were immunized with ADS-MVA. Two mice, M260 and M262, were given 2×10^6 $TCID_{50}$ of the vaccine, and the other six mice, M263, M264, MM1, MM2, MM3, and MM4, received 2×10^7 $TCID_{50}$. All of the animals were immunized i.m. twice with a 3-week interval. Serum samples were collected and subjected to the neutralization assay 2 weeks after the second immunization. As controls, four mice (M265, M266, M267, and M268) received ADC-MVA, which expresses HCV E1E2 protein. The vaccine dose used for the control group was 2×10^7 $TCID_{50}$ per mouse. We also included two mice (N1 and N2) that only received saline injections as background controls. As depicted in Fig. 2, high levels of neutralizing antibodies were induced in eight mice that were immunized with ADS-MVA. Fifty percent of the virus was inhibited or neutralized (i.e., the 50% inhibitory concentration [IC_{50}]) after the sera were diluted by >1,000-fold in the high-dose group. In contrast, two groups of control mice did not yield any neutralizing antibodies. Moreover, there was a clear dose dependency between the two ADS-MVA dosing groups. Therefore, ADS-MVA induces potent SARS-CoV-specific neutralizing antibodies in mice.

Second, to find out whether similar antibodies could be induced in a second animal species, we immunized two rabbits with ADS-MVA. The immunization schedule was slightly different for the time of the second immunization, which was done after a 4-week interval. Considering the body size of the rabbits, we used a higher dose of 10^8 $TCID_{50}$ for each i.m. immunization. The dosing selection was based on our previous experience in testing an MVA-based human immunodeficiency virus type 1 vaccine in small animals. Again, SARS-CoV-specific antibodies were induced at high levels in two rabbits R524 and R525 who received ADS-MVA (Fig. 3). The IC_{50} was achieved when the sera were diluted ~10,000-fold. Two control rabbits R520 and R521 who received the ADC-MVA did not produce any measurable SARS-CoV neutralizing antibodies. These data have demonstrated that the high level of neutralizing antibodies induced by ADS-MVA is not a species-

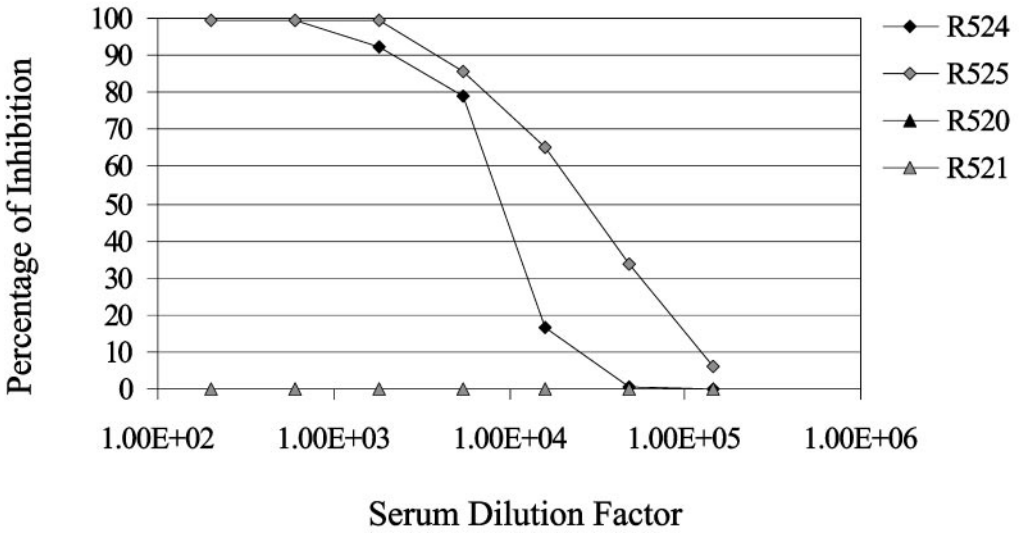

FIG. 3. Anti-SARS-CoV specific neutralization antibody response in New Zealand White rabbits after ADS-MVA vaccinations. Each rabbit was designated with a number such as R524. The animals were immunized i.m. twice, 4 weeks apart, with 10^8 $TCID_{50}$ of ADS-MVA per animal (R524 and R525) or 10^8 $TCID_{50}$ of ADC-MVA (R520 and R521).

specific phenomenon. Of note, all immunized small animals tolerated the vaccine very well. No evident disease or body weight loss was observed during the experimental period.

Immunogenicity of ADS-MVA in rhesus monkeys. To explore the potential use of ADS-MVA in humans, we have further tested ADS-MVA in rhesus monkeys. A total of eight rhesus monkeys were included in the present study, four vaccinated with ADS-MVA and another four vaccinated with ADC-MVA. Similar to the immunization scheme in rabbits, the monkeys were vaccinated twice within a 4-week interval. We used a dose of 1×10^8 $TCID_{50}$ for the first immunization and a dose of 3×10^8 $TCID_{50}$ for the second injection. As for the small animals, the route of immunization was i.m. injection. Sequential blood samples were collected to measure the presence of neutralizing antibodies. The titer of neutralizing antibodies was low in all four monkeys 2 weeks after the first immunization (Fig. 4A). However, the IC_{50} titer was significantly boosted for 100-fold or higher after the second immunization (Fig. 4B). These data indicate the need of a second immunization for inducing high levels of neutralizing antibodies. In controls, none of the ADC-MVA-immunized monkeys yielded detectable levels of SARS-CoV neutralizing antibodies. Therefore, ADS-MVA can induce high level of SARS-specific neutralizing antibodies in rhesus monkeys in addition to small animals. Since SARS-CoV transmission targets the respiratory system, we also tested lung and bronchus lavage samples for neutralizing antibodies. The samples were collected at the time the blood specimens were obtained. However, no detectable amount of neutralizing antibody activity was found in all eight monkeys when the samples were tested, even at the lowest dilution ($<$1:10) (data not shown).

Mapping of a neutralizing epitope region. Since potent neutralizing antibodies were induced in animals by using full-length S glycoprotein, it is crucial to further map the region where the neutralizing epitope region resides. For this purpose, we generated a series of DNA vaccines by preserving the amino acids from the N-terminal of the S protein, including S200, S400, S600, and S800, respectively (Fig. 5A). For better expression in mammalian cells, we engineered a synthetic S gene with an optimized codon sequence. Moreover, the leader sequence of the S gene was replaced with a tissue plasminogen activator gene leader sequence. This leader sequence was previously shown to enhance protein expression by facilitating transport of the protein from the endoplasmic reticulum to the Golgi apparatus (17, 24, 48). We were able to express these fragmented proteins in human 293T cells, as demonstrated by a Western blot analysis (Fig. 5B). To ensure the induction of antibody response, we adapted an in vivo electroporation technique for the delivery of the DNA plasmid into the rabbits (3, 49). Two rabbits per group received DNA vaccinations twice with a 3-week interval. Four rabbits that received S200 (R416 and R417) or S400 (R418 and R419) produced binding antibodies, as determined by Western blot analysis. These antibodies, however, had no neutralizing activity. In contrast, neutralizing antibodies were detected in all four rabbits when S600 (R681 and R682) and S800 (R683 and R684) were used as the immunogen (Fig. 6). Two control rabbits (R518 and R519) immunized with DNA expressing HCVE1E2 had no detectable neutralizing antibodies. These findings indicate that a neutralizing epitope region was determined by amino acids between 400 to 600 amino acids counting from the N-terminal of the S protein. Interestingly, this region overlaps with the receptor ACE2-binding region (S318-510), which has been recently determined (50).

The ACE2-binding region contains a major neutralizing domain within the full S protein. Since the newly identified region overlaps the ACE2-binding region, we further determined whether the latter harbors a major neutralizing domain. For this purpose, we performed two antibody absorption experiments. In one experiment, the soluble RBR-Fc was used to absorb the antibodies in sera which specifically bind to RBR of the fusion protein. Then, the antibody-fusion protein complex

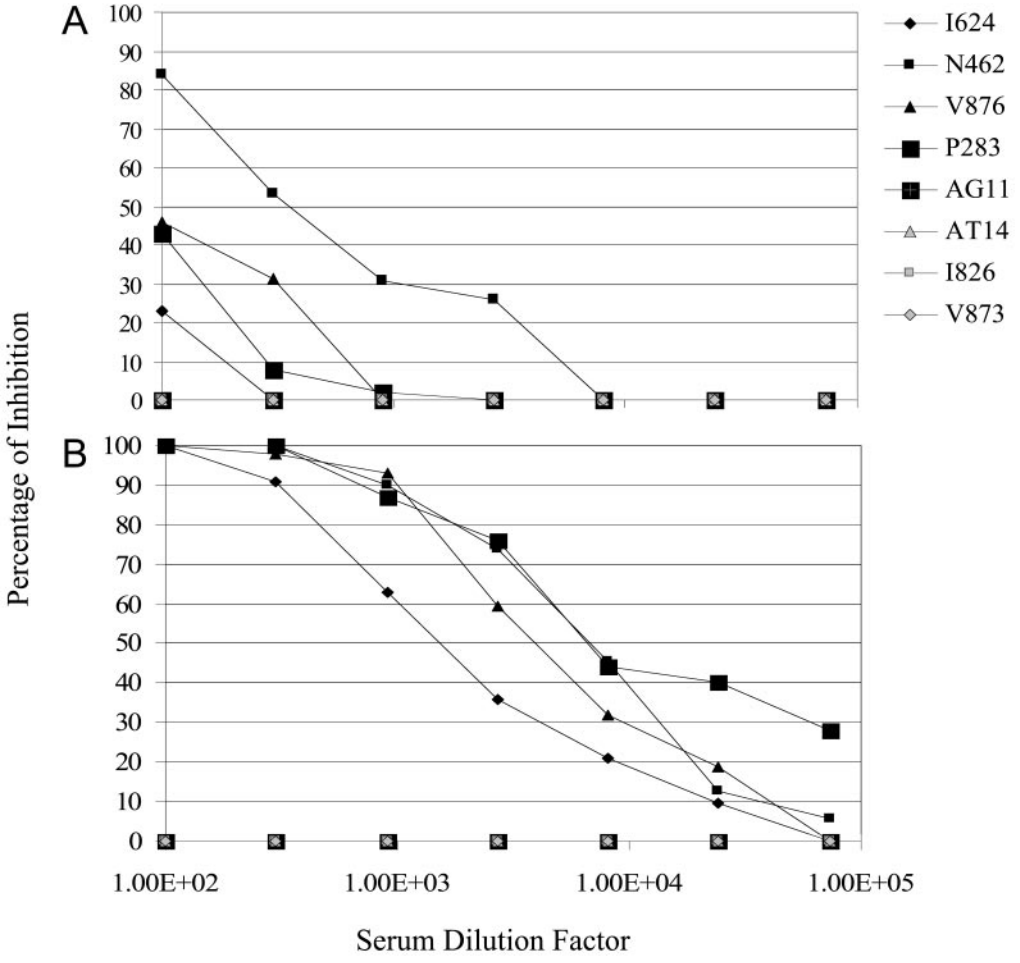

FIG. 4. Anti-SARS-CoV-specific neutralization antibody response in Indian rhesus macaques after ADS-MVA vaccinations. Four animals (I624, N462, V876, and P283) were immunized i.m. twice, 4 weeks apart, with 1×10^8 and 3×10^8 $TCID_{50}$ of ADS-MVA per animal, respectively. Another four macaques (AG11, AT14, I826, and V873) were treated the same but with ADC-MVA. (A) Neutralizing antibody responses after the first immunization. (B) Boosted neutralizing antibody responses after the second immunization.

was removed by carrier agarose beads which were coated with anti-human Fc antibodies. This assay was used to test immune sera from rabbits and mice because their immunoglobulin sera do not cross-react with the anti-human Fc antibodies coated on the carrier agarose beads. The rabbit and mouse immune sera were diluted (1:300 to 1:600) based on the results of neutralization assay so that no excessive amount of neutralizing antibodies would interfere with the test. As shown in Fig. 7A, soluble RBR-Fc was able to absorb and remove the majority of neutralizing antibodies in the rabbit immune sera, ranging from 52% (R682.M) to 100% (R681.D and R682.D). Sera R681.D and R682.D were collected after rabbits 681 and 682 received two DNA vaccinations, whereas sera R681.M and R682.M were from the same animals after two additional ADS-MVA vaccinations. As controls, beads alone in RBR-Fc(−) groups had no effects on neutralizing antibodies. Interestingly, the neutralizing activity was almost completely removed from the R681.D and R682.D sera, which were harvested after two S600 DNA immunizations. In contrast, the remaining sera maintained some residual activity (20 to 48%). These remaining sera were harvested after two ADS-MVA immunizations (R524.M and R525.M) or two additional ADS-MVA immunizations (R681.M and R682.M). Similar findings were made when ADS-MVA immunized mouse sera were tested by using the same assay (Fig. 7B). Again, the majority (65 to 95%) of the mouse neutralizing antibodies were absorbed and removed by using the RBR-Fc recombinant protein. Therefore, the data indicate that there is a predominant neutralizing domain located in the ACE2-binding region despite ADS-MVA tends to induce relatively broader neutralizing activity in comparison to S600 DNA vaccine. It is possible that there are some other neutralizing epitopes that were presented by the full-length S protein but absent in the S600-based immunogen.

In the second experiment for testing monkey sera, RBR-Fc-conjugated Sepharose 4 Fast-Flow beads were used to absorb and remove monkey antibodies that bound to the RBR portion. As shown in Fig. 7C, unconjugated beads, RBR-Fc(−), had no effect on the activity of neutralizing antibodies. In contrast, conjugated beads, RBR-Fc(+), were able to remove the majority of neutralizing antibodies (>80%) in monkey sera similar to the findings with rabbit and mouse sera. Taking

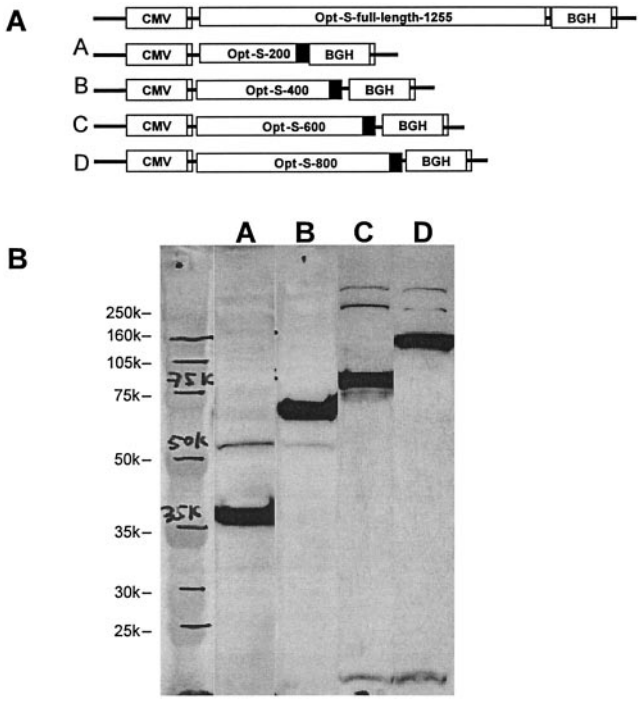

FIG. 5. Construction and expression of humanized fragmented S gene in the context of DNA vaccines. (A) Four S gene fragments (labeled A to D) were each constructed under cytomegalovirus (CMV) promoter in mammalian expression vector pcDNA3.1. (B) Fragmented S proteins of corresponding lengths were expressed in 293T cells in a Western blot analysis.

TABLE 1. Virus detection in Chinese macaques after challenge with SARS-CoV

Macaque	NAb titer (IC_{50})[a]	Viral isolation[b]	RT-PCR at day[c]:		
			2	4	6
ADS-MVA					
Rh0412	2,259	−	−	−	−
Rh0413	1,523	−	+	−	−
Rh0416	3,634	−	−	−	−
Rh0417	9,531	−	−	−	−
Control MVA					
Rh0420	0	−	+	+	+
Rh0421	0	+	+	+	+
Rh0424	0	−	−	+	+
Rh0425	0	+	−	+	+

[a] Neutralizing antibodies were detected at the time just before the viral inoculation. NAb, neutralizing antibody.
[b] Virus isolation was attempted by using lung tissue homogenates. +, positive; −, negative.
[c] RT-PCR was performed on nasopharyngeal swab specimens. +, positive; −, negative.

together our results, we conclude that ADS-MVA induced neutralizing antibodies primarily target on the ACE2-binding region of SARS-CoV. This targeted interaction is shared by three different animal species which indicated the lack of a species restriction for the function of neutralizing antibodies generated by ADS-MVA.

Protection of ADS-MVA immunized Chinese rhesus monkeys from SARS-CoV challenge. After two immunizations of four Chinese rhesus monkeys with ADS-MVA via i.m. and i.n. routes, high levels of SARS-CoV specific neutralizing antibodies were induced in all four animals. At the time of virus challenge, the IC_{50} values of these antibodies still maintained with reciprocal serum dilution over 1500-fold (Table 1). After

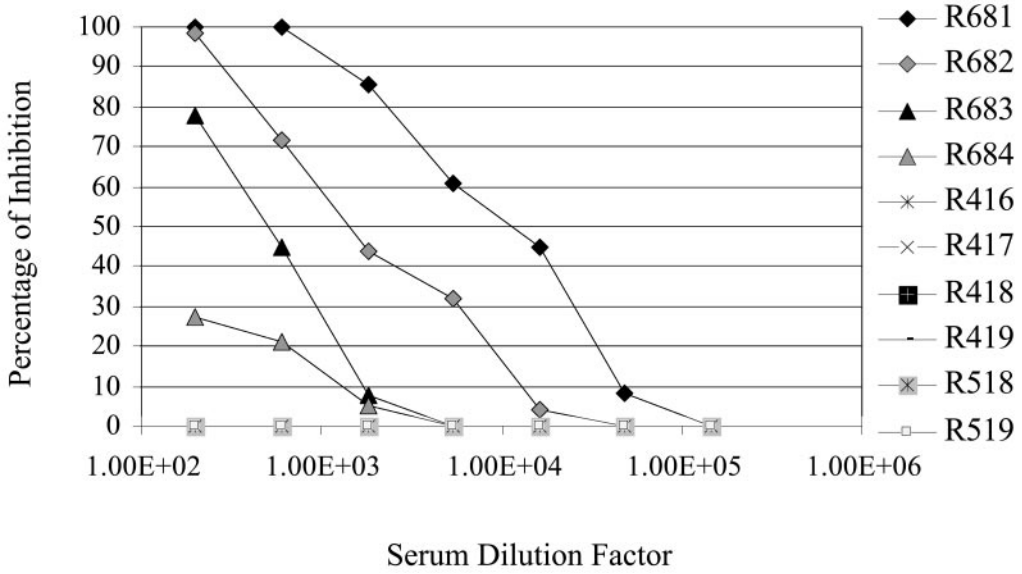

FIG. 6. Anti-SARS-CoV-specific neutralization antibody response in New Zealand White rabbits after DNA vaccinations. Two animals per group were immunized twice, 4 weeks apart, using an in vivo electroporation technique with DNA vaccines S200 (R416 and R417), S400 (R418 and R419), S600 (R681 and R682), and S800 (R683 and R684). Another two rabbits were vaccinated with a DNA vaccine expressing HCVE1E2 gene (R518 and R519).

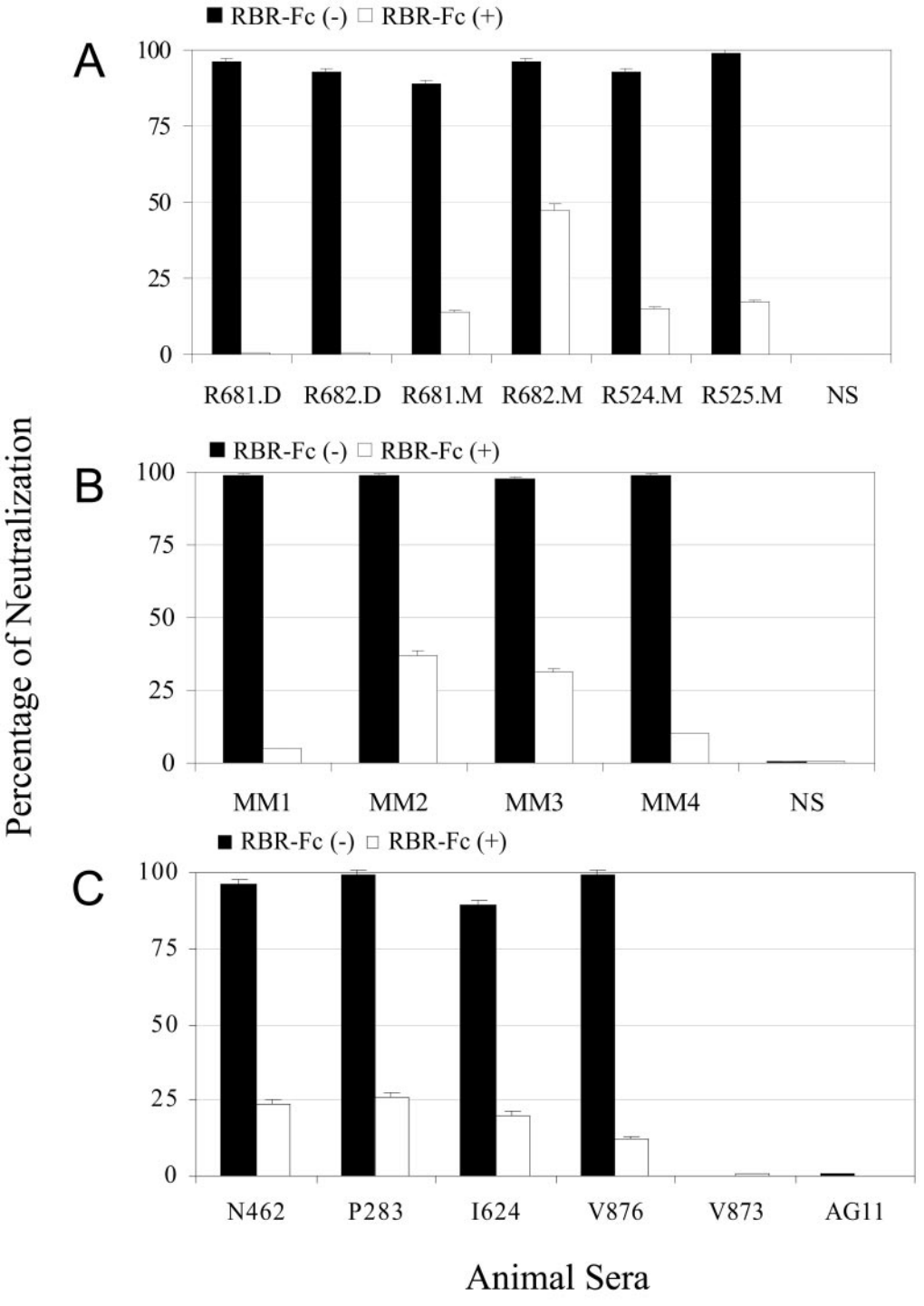

FIG. 7. Absorption and removal of anti-SARS-CoV-specific neutralization antibodies of New Zealand White rabbits (A), BALB/c mice (B), and monkeys (C) by an ACE2 receptor-binding region and human Fc fusion protein (RBR-Fc). For rabbit and mouse sera, the neutralization antibody–RBR-Fc complex was removed by agarose beads that were precoated with specific anti-human Fc antibodies. The anti-human Fc antibodies do not cross-react with rabbit or mouse immunoglobulin but do cross-react with monkey samples. Therefore, for monkey sera, neutralization antibodies were removed by beads directly conjugated with RBR-Fc. In each experiment, the corresponding beads controls did not affect the activity of neutralization antibodies, as presented in groups of RBR-Fc(−). The experiment was carried out in triplicates, and the average values are presented. The error bars stand for the standard deviation.

virus challenge, SARS-CoV shedding detected by RT-PCR was only detected in the nasopharyngeal specimens of one of the four ADS-MVA immunized animals (Rh0413) on day 2 after virus challenge. No virus shedding was detectable on days 4 and 6 postchallenge in these four macaques. In contrast, two of four control ADC-MVA-immunized macaques were RT-PCR positive on day 2 postchallenge. Moreover, all four of these macaques were RT-PCR positive on days 4 and 6 postchallenge, suggesting continuous virus shedding. In addition, SARS-CoV was isolated from the lung specimens of two of four control macaques but not from ADS-MVA-immunized macaques on day 7 postchallenge. These data indicate that immune responses induced by ADS-MVA very likely protected Chinese rhesus monkeys from SARS-CoV challenge.

DISCUSSION

In this study, live attenuated MVA was used as a vector to construct a SARS S-protein-based vaccine. The newly constructed recombinant ADS-MVA tolerated the insertion of full-length SARS S protein under the strong synthetic promoter (Fig. 1). ADS-MVA was genetically stable and was able to replicate in CEF cells to high titers (10^8 to 10^9 PFU/ml). Moreover, since ADS-MVA did not spread in mammalian cells tested, we believe that this new recombinant virus has maintained the attenuated growth phenotype as the parental MVA and therefore could potentially serve as a safe vaccine for human and animal use. Our vaccine offers a great advantage over the use of live attenuated and killed SARS-CoV as vaccines because the latter pose a high risk to laboratory and vaccine production workers and also to vaccinees if the virus inactivation is incomplete.

ADS-MVA induces high levels of neutralization antibodies in animals without species restriction. Recent findings that neutralizing antibodies to S protein confer protective immunity in various animal species have shed light on SARS vaccines primarily targeting the humoral immune response (4, 6, 46, 55). In the present study, high levels of neutralizing antibodies were induced by ADS-MVA vaccination in three animal species: mice, rabbits, and monkeys. The IC_{50} was achieved in most animals after two ADS-MVA vaccinations with the reciprocal dilution titer of sera reached between 1,000 and 10,000. Importantly, the neutralizing antibodies induced were SARS-specific because animals immunized with recombinant MVA carrying HCV genes did not have detectable responses. The high titer of the neutralizing antibodies also indicated that the SARS S protein has been expressed in vivo and was properly presented for immune recognition. Our immunogenicity data in monkeys indicated that a similar level of neutralizing antibodies could be induced in primate species by using ADS-MVA. Therefore, consistent with previous findings, vaccinia virus MVA is a good live virus vector system for inducing potent humoral response.

Inoculation i.m. does not seem to induce detectable amount of neutralizing antibodies in the respiratory system. There have been a number of issues that needed to be addressed related to inducing protective immunity in animals. The route of immunization is one of such issues. Two recent studies, one using DNA and the other using MVA-based vaccine, demonstrated protection against SARS-CoV infection in mice via i.m. immunization (4, 55). In the latter study, i.n. immunization also induced similar protection. Although the level of neutralizing antibodies in the respiratory system was not demonstrated in these studies, the vaccine-induced protection was very successful. In the present study, we did not find detectable amounts of neutralizing antibodies in the respiratory system of immunized monkeys despite the high antibody titer in the serum. If the failure of detection is not due to the sensitivity of our assay system, it is crucial to evaluate the role of mucosal immunity in preventing SARS-CoV infection in the context of various routes and types of vaccination in monkeys.

In our SARS-CoV challenge study with Chinese rhesus monkeys, we used both i.m. and i.n. immunization routes. We used Chinese rhesus monkeys because they are susceptible to SARS-CoV infection. Moreover, since the genetic background of monkeys is quite different from the inbred mouse model system, the use of Chinese rhesus monkeys would help us to determine whether or not ADS-MVA can induce protective immunity in primate species. This is a big public health concern, especially when a preliminary finding showed that prevaccinated ferrets displayed vaccine-enhanced infection after they were exposed to SARS-CoV (14). Our vaccination procedure was successful because a high level of neutralizing antibodies was induced in all four ADS-MVA vaccinated monkeys at the time of virus challenge. In comparison to the control ADC-MVA immunized macaques, no SARS-CoV could be isolated from the lungs of ADS-MVA-immunized macaques. Moreover, no SARS-CoV shedding was detected in the upper respiratory tract of these animals by RT-PCR after 2 days of virus challenge. We did not see ADS-MVA-enhanced infection after the macaques were exposed to SARS-CoV. Our data suggested that a vaccine-induced immune response very likely protected Chinese macaques from SARS-CoV challenge. However, more detailed studies are planned to investigate the viral pathogenesis in the lungs of these animals. We also need to address the pathological, serological, and virologic outcomes in experimental animals beyond day 7 of virus challenge.

A major neutralizing epitope resides in the receptor ACE2-binding region of the SARS S protein. In the present study, we have mapped a neutralizing epitope-containing region by using fragmented S genes as immunogens. The region mapped (S400-600) overlaps with the 193-amino-acid fragment of the S protein, an ACE2 RBR (S318-510) (50). Interestingly, the mapped region also overlaps with a domain (S261-672) containing neutralizing epitope 80R of a human monoclonal antibody (42). These observations guided us to further determine the significance of the overlap in relation to its biological function. Using a recombinant RBR-Fc protein as a tool, we were able to remove the majority of the neutralizing antibodies in three animal species induced by ADS-MVA. On the one hand, our findings indicate the identification of a major neutralizing epitope located within the ACE2-binding region of SARS S protein. On the other hand, the data generated by the neutralization assay also suggested a major mechanism for the S-protein-based vaccine. This mechanism is likely that the S-protein-based vaccine tends to induce potent neutralizing antibodies that primarily prevent the virus to enter target cells by binding to the virions to block their interaction with ACE2. Despite this mechanism, however, there might be other neu-

tralizing epitopes outside the dominant epitope in the ACE2-binding region. It was evident in our experiments that incomplete absorption was often seen with sera derived from animals immunized with full S protein. This evidence supports recent findings that more than one neutralizing epitope was identified on the S protein (58, 59). Therefore, using the complete S protein as immunogen may be superior to the fragmental S vaccine candidates.

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