Cross-Protection against Marburg Virus Strains by Using a Live, Attenuated Recombinant Vaccine

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Marburg virus (MARV) has been associated with sporadic episodes of hemorrhagic fever, including a recent highly publicized outbreak in Angola that produced severe disease and significant mortality in infected patients. MARV is also considered to have potential as a biological weapon. Recently, we reported the development of a promising attenuated, replication-competent vaccine against MARV based on recombinant vesicular stomatitis virus (VSV) expressing the glycoprotein of the Musoke strain of MARV (VSVΔG/MARV GP-Musoke). We used this vaccine to demonstrate complete protection of cynomolgus monkeys against a homologous MARV challenge. While these results are highly encouraging, an effective vaccine would need to confer protection against all relevant strains of MARV. Here, we evaluated the protective efficacy of the VSVΔG/MARVGP-Musoke vaccine against two heterologous MARV strains, the seemingly more pathogenic Angola strain and the more distantly related Ravn strain. In this study, seven cynomolgus monkeys were vaccinated with the VSVΔG/MARVGP-Musoke vector. Three of these animals were challenged with the Angola strain, three with the Ravn strain, and a single animal with the Musoke strain of MARV. Two animals served as controls and were each injected with a nonspecific VSV vector; these controls were challenged with the Angola and Ravn strains, respectively. Both controls succumbed to challenge by day 8. However, none of the specifically vaccinated animals showed any evidence of illness either from the vaccination or from the MARV challenges and all of these animals survived. These data suggest that the VSVΔG/MARVGP-Musoke vaccine should be sufficient to protect against all known MARV strains.

Marburg virus (MARV) causes severe and often fatal infections in humans and nonhuman primates. Historically, several strains of MARV have produced confirmed case fatality rates ranging from 23% to slightly greater than 50% (4, 16). However, in 2004 and 2005 a new strain of MARV caused even more significant mortality during a large outbreak in Angola. Case fatality rates during this episode fluctuated around 90%. An initial report by the World Health Organization noted that the epidemic killed 329 people of the 374 that were infected; the outbreak was recently declared over, and these numbers were revised, with the updated report noting that there were 227 deaths among the 252 reported cases (12). The reasons for the increased lethality of this new strain of MARV are presently unknown but are of significant concern.

While there are currently no licensed vaccines or antivirals to prevent or treat MARV infections, we recently described the development of a promising new replication-competent vaccine against MARV based on recombinant vesicular stomatitis virus (VSV) (7, 14). In one study, we demonstrated complete protection of cynomolgus macaques against a high-dose (1,000 PFU) lethal MARV challenge by use of a single injection of recombinant VSV vectors expressing the glycoprotein (GP) of the homologous Musoke strain of MARV (MARV-Musoke) (14). More recently, we demonstrated that the same vaccine vector used as postexposure treatment for rhesus monkeys (Macaca mulatta) infected with 1,000 PFU of strain Musoke was also able to protect all animals from clinical disease and death (5).

MARV and Ebola virus (EBOV) comprise the two genera that make up the family Filoviridae (6). There is a single species, Lake Victoria marburgvirus, within the MARV genus. There are a number of different strains of MARV. Comparative analyses of the GP and viral protein (VP) 35 genes of MARV strains showed that there are two distinct lineages within the Lake Victoria marburgvirus species of MARV. The original MARV isolates from the 1967 episodes in Marburg, Germany (Popp and Ratayczak strains), from a case in 1975 in South Africa (Ozolin strain), and from 1980 in Kenya (Musoke strain) comprise one lineage. An isolate from Kenya in 1987 (Ravn strain) represents a second genetic lineage within the Lake Victoria marburgvirus species of MARV (21 to 25% amino acid difference) (21).

MARV is composed of seven structural proteins and the nonsegmented negative-sense viral RNA genome. Four proteins (NP, VP35, VP30, and L) make up the helical nucleocapsid, which is surrounded by a matrix that is composed of the...
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viral proteins VP40 and VP24. The surface of MARV virions is coated with spikes that consist of the structural GP. The GP plays a role in virus entry and pathogenesis and serves as a major and logical target for vaccine strategies, including our recombinant VSV-based system (14).

While most strains of MARV produce lethal infections in nonhuman primates, the disease courses vary among the strains and are in general more protracted than what is seen with EBOV. Recent studies have suggested that the new Angola isolate of MARV appears to produce a disease in nonhuman primates that is more rapid and severe than that produced by other MARV strains and, in fact, appears to be as virulent as that produced by Zaire ebolavirus (ZEOBV) in rhesus macaques (T. W. Geisbert, unpublished observation).

In developing vaccines against any virus, there are always concerns about broad protection and the ability to protect against different strains or isolates of the same virus. Of particular concern regarding cross-protection among the MARV strains is results reported using a different vaccine vector system. Notably, a study using a platform based on Venezuelan equine encephalitis virus (VEEV) replicons showed that cynomolgus monkeys vaccinated with VEEV (MARV GP) or VEEV (MARV NP) replicons based on strain Musoke were protected against lethal homologous challenge (9) but not against a challenge with the Ravn strain of MARV (MARV-Ravn) (10). This result raises the concern that the 21 to 23% difference in amino acids between the two MARV lineages may be significant enough to affect the ability of a candidate vaccine to confer cross-protection against the different MARV strains. Here, we tested the ability of our recombinant VSV vaccine expressing the MARV-Musoke strain GP to protect nonhuman primates against a lethal challenge with either the more genetically diverse Ravn strain or the seemingly more pathogenic Angola strain (MARV-Angola).

MATERIALS AND METHODS

Vaccine vectors and viruses. The recombinant VSVs expressing the GPs of ZEOBV (VSVΔG/ZEOBGVP) and MARV-Musoke (VSVΔG/MARVGP-Musoke) were generated by use of the infectious clone for the VSV Indiana serotype as described recently (7, 14). Briefly, the appropriate open reading frames (ORFs) for the GPs were generated by PCR, cloned into the VSV genomic vectors lacking the VSV GP gene, sequence confirmed, and originally rescued using the method described earlier. MARV-Musoke was isolated from a human case in 1980 in Kenya (22), MARV-Ravn was isolated from a human case in 1987 in Kenya (13), and MARV-Angola was isolated from a patient of the recent outbreak in Angola in 2005 (26).

Sequencing and phylogenetic analysis. MARV-Angola was isolated from clinical specimens (whole blood) from several patients. The sequence of the GP gene was determined using primers based on the GP sequences of the Musoke strain. The deduced amino acid sequence of the Angola GP ORF was compared with the amino acid sequences of different filovirus GPs which were retrieved from GenBank. Phylogenetic analysis was performed with MEGA version 3.1 (www.megasoftware.net) using a neighbor-joining tree and 1,000 bootstrap replicates.

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ulations were further defined by anti-CD4 and anti-CD8 positivity, respectively. Cytokine-positive cells were defined as percentages within these individual lymphocyte subsets, and at least 200,000 events were analyzed for each sample.

RESULTS

Sequence analysis. At the beginning of this study, the GP sequence of MARV-Angola was not determined. Therefore, we sequenced the GP ORF of several isolates which were obtained in Winnipeg, Manitoba, Canada, by inoculation of Vero E6 cells with whole blood collected from patients during the MARV-Angola outbreak. Comparative analysis showed that all GP gene sequences were identical. Thus, the deduced amino acid sequence of the GP ORF of one of the isolates was used for phylogenetic analysis with the deduced amino acid sequences of several EBOV and MARV GPs, including two recently deposited sequences from the MARV outbreak in Durba/Watsa, Democratic Republic of the Congo (Fig. 1). The phylogenetic analysis demonstrated that MARV strains separated into two major branches. One branch included strains from 1987 (Ravn) and 1998 to 2000 (Durba/Watsa). The second major branch again separated into two further branches, with strains from 1975 (Ozolin) and 1998 to 2000 (Durba/Watsa) separated from strains originating in 1967 (Popp and Ratayczak), 1980 (Musoke), and 2004 to 2005 (Angola). The close relationship between the Angola and Musoke strains indicated a likelihood of cross-protection by the VSVΔG/MARVGP-Musoke vaccine against a heterologous challenge with the Angola strain. However, cross-protection against a challenge with the more distantly related Ravn strain seemed less likely. We recently demonstrated that the VSVΔG/MARVGP-Musoke vaccine does not provide any cross-protection against ZEBOV strains (14).

Clinical observations. A total of nine cynomolgus monkeys were used to evaluate whether a preventative vaccination strategy employing a single injection of VSVΔG/MARVGP-Musoke could cross-protect against MARV hemorrhagic fever caused by the Ravn and Angola strains (Fig. 2A). We vaccinated seven animals by intramuscular injection of VSVΔG/MARVGP-Musoke vectors (subjects no. 1 to 7) and two control animals with nonspecific VSVΔG/ZEBOVGP (controls no. 1 and 2). Animals were challenged 28 days after the single-dose vaccine with either heterologous MARV-Ravn (subjects no. 1 to 3 and control no. 1) or heterologous MARV-Angola (subjects no. 4 to 6 and control no. 2). One animal (subject no. 7) was challenged with homologous MARV-Musoke and served as an internal vaccine control, as we have shown previously that the VSVΔG/MARVGP-Musoke vaccine can provide complete protection against a homologous MARV challenge (14). Animals were monitored closely after both vaccination and MARV challenge for clinical symptoms of illness, viremia from either the vaccine or MARV, and shedding of the recombinant VSVs (Fig. 2A). None of the animals vaccinated with VSVΔG/MARVGP-Musoke showed any evidence of clinical illness either after vaccination or after the MARV challenge. Importantly, all of the animals vaccinated with VSVΔG/MARVGP-Musoke showed any evidence of clinical illness either after vaccination or after the MARV challenge. Importantly, all of the animals vaccinated with VSVΔG/MARVGP-Musoke survived against a heterologous challenge with either MARV-Ravn or MARV-Angola with no observable clinical
changes. In contrast, both the MARV-Ravn-challenged (control no. 1) and the MARV-Angola-challenged (control no. 2) control animals followed a typical disease course, developing fevers, macular rashes, and signs of depression by day 6 postinfection, and succumbed to MARV hemorrhagic fever on day 8 postinfection (Fig. 2B).

**Viremia and blood chemistry.** To determine whether viremia or shedding of the recombinant VSVs occurred after immunization, whole-blood and swab samples from all nine of the vaccinated animals were analyzed by RT-PCR and virus isolation. A transient and low-level \( \leq 1.7 \log_{10} \text{PFU/ml} \) recombinant VSV viremia was detected by virus isolation at day 3 after vaccination in plasma from four of the VSV/ΔG/EBOVGP vaccinated animals (subjects no. 1, 4, 5, and 7) (Fig. 3A). Also, a low level of VSV/ΔG/ZEBOVGP was detected by virus isolation from a nasal swab of one of the control animals at day 3. However, this animal had evidence of self-inflicted bleeding around the nares so we are uncertain as to the exact significance of this particular finding. In addition, we did not detect VSV/ΔG/ZEBOVGP in plasma of this control animal by virus isolation, further questioning the importance of the low level of recombinant virus detected from the nasal swab.

Blood samples were also analyzed after MARV challenge for evidence of MARV replication and shedding by plaque assay (Fig. 3B) and by RT-PCR (data not shown). By day 6, both the MARV-Ravn-challenged control animal (control no. 1) and the MARV-Angola-challenged control animal (control no. 2) developed high MARV titers in the blood as detected by plaque assay \( (>10^7 \log \text{PFU/ml}) \). RT-PCR was more sensitive and showed evidence of MARV in plasma of these two control animals by day 3 postinfection. In contrast, no MARV was detected in the plasma by virus isolation or RT-PCR in any of the animals vaccinated with VSV/H9004G/MARVGP-Musoke.

Analysis of blood chemistry and hematology was performed before and after the MARV challenges. Again, in accordance with no clinical symptoms, none of the animals vaccinated with VSV/ΔG/MARVGP-Musoke showed any evidence of changes in blood chemistry or hematology after vaccination or after the MARV challenges. In contrast, while neither of the control animals showed any evidence of changes in blood chemistry or hematology after vaccination with VSV/ΔG/ZEBOVGP, both controls developed lymphopenia and thrombocytopenia after the MARV challenges. In addition, both of these controls showed significant elevations in circulating levels of enzymes (alkaline phosphatase, aspartate aminotransferase,
and alanine aminotransferase) associated with impairment of the liver by day 6 postinfection.

**Evaluation of antibody and cellular immune responses.** The antibody responses of the cynomolgus macaques immunized with VSVΔG/MARVGP-Musoke were evaluated after vaccination (day −14 and day 0) and after MARV challenge (day 14 and day 28) by IgG ELISA and by plaque neutralization tests (PRNT<sub>50</sub>). All of the animals developed high anti-MARV IgG...
antibody levels (≥1:1,000) by the day of the MARV challenges (day 0) (Fig. 4A). Low levels of anti-MARV neutralizing antibodies (1:10 to 1:20) were observed for four of the seven animals vaccinated with VSVΔG/MARVGP-Musoke at the day of MARV challenge (Fig. 4B). All seven of these animals developed low levels of neutralizing antibodies (1:10 to 1:80) by day 28 after the MARV challenges (Fig. 4B).

To better understand the cellular responses of T-cell popu-
lations found in peripheral blood mononuclear cell fractions of specifically and nonspecifically vaccinated animals in mediating protection against MARV challenge, flow cytometry was employed during the course of study. There was no evidence of either gamma interferon or tumor necrosis factor alpha production in CD4 or CD8 T-cell populations either before or after the MARV challenges in any of the animals used in this study (data not shown).

DISCUSSION

The recent outbreak of MARV in Angola generated significant interest from the popular press and reinforces the danger of emerging viruses, such as MARV, as significant public health threats. However, MARV also poses a threat as a potential biological weapon (1). It is primarily for this reason that there has been an increased investment in developing countermeasures against this highly lethal pathogen.

Significant advances in developing countermeasures against the filoviruses, particularly regarding the creation of promising vaccines, have been made over the last decade (14, 23, 24). Recently, we showed that a replication-competent vaccine based on recombinant VSV expressing either the GP of EBOV or the GP of MARV could completely protect nonhuman primates against a homologous filovirus challenge (14). While the EBOV vaccine based on the Zaire species GP completely protected macaques against a lethal ZEBOV challenge, it was unable to protect macaques against challenge with another EBOV species, SEBOV. This was not an unexpected finding since macaques that survive experimental challenge with wild-type SEBOV are not protected against a subsequent back-challenge with ZEBOV (2). Indeed, there is a 37 to 44% difference between SEBOV and ZEBOV at the nucleotide and amino acid levels (20), further supporting the view that there would be little if any cross-protection among these species of EBOV.

From the perspective of vaccine development, it is apparent that a vaccine that would protect against both SEBOV and ZEBOV would likely need to include SEBOV-specific antigens as well as ZEBOV-specific antigens. While there is only a single species of MARV, as noted previously, there are two genetically disparate lineages of MARV with 21 to 23% difference in amino acids (21). This variation raised concern that there would be little if any cross-protection among these species of EBOV.

Whether these vectors can confer protective immunity against highly lethal pathogens, such as the hemorrhagic fever viruses, and whether there will be any trade-off regarding durability for potential safety remain to be determined. Not only have live, attenuated VSV-based vaccines shown promise in nonhuman primate models of EBOV and MARV infections, we also recently demonstrated the success of this platform in protecting monkeys against a lethal Lassa virus challenge (8). Others are using replication-competent VSV-based systems as candidate vaccines for a variety of viruses, including human immunodeficiency virus (HIV). In fact, a candidate recombinant VSV-based HIV vaccine was shown previously to prevent AIDS-like disease in monkeys (19). Moreover, in an effort to address safety concerns with live vaccines, Wyeth is currently engineering VSV vectors that are highly attenuated in animals. The company plans to begin human trials with these vectors expressing HIV antigens that are highly attenuated in animals. The company plans to begin human trials with these vectors expressing HIV antigens that are highly attenuated in animals. The company plans to begin human trials with these vectors expressing HIV antigens that are highly attenuated in animals. The company plans to begin human trials with these vectors expressing HIV antigens that are highly attenuated in animals.

The use of attenuated recombinant VSV-based vectors has proven to be an effective and promising platform for the development of preventive vaccines against a number of pathogenic viruses. Findings from the current study taken together with observations from our previous work (5, 14) suggest that a vaccine that would confer protection against all relevant
species or strains of filoviruses would likely require three antigens, including those specific for the ZEBOV GP, the SEBOV GP, and the MARV-Musoke GP.

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REFERENCES


