Postexposure protection against Marburg haemorrhagic fever with recombinant vesicular stomatitis virus vectors in non-human primates: an efficacy assessment

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Summary

Background **Effective countermeasures are urgently needed to prevent and treat infections caused by highly pathogenic and biological threat agents such as Marburg virus (MARV). We aimed to test the efficacy of a replication-competent vaccine based on attenuated recombinant vesicular stomatitis virus (rVSV), as a postexposure treatment for MARV haemorrhagic fever.**

Methods **We used a rhesus macaque model of MARV haemorrhagic fever that produced 100% lethality. We administered rVSV vectors expressing the MARV Musoke strain glycoprotein to five macaques 20–30 min after a high-dose lethal injection of homologous MARV. Three animals were MARV-positive controls and received non-specific rVSV vectors. We tested for viraemia, undertook analyses for haematology and serum biochemistry, and measured humoral and cellular immune responses.**

Findings **All five rhesus monkeys that were treated with the rVSV MARV vectors as a postexposure treatment survived a high-dose lethal challenge of MARV for at least 80 days. None of these five animals developed clinical symptoms consistent with MARV haemorrhagic fever. All the control animals developed fulminant disease and succumbed to the MARV challenge by day 12. MARV disease in the controls was indicated by: high titres of MARV (10^3–10^5 plaque-forming units per mL); development of leucocytosis with concurrent neutrophilia at end-stage disease; and possible damage to the liver, kidney, and pancreas.**

Interpretation **Postexposure protection against MARV in non-human primates provides a paradigm for the treatment of MARV haemorrhagic fever. Indeed, these data suggest that rVSV-based filoviral vaccines might not only have potential as preventive vaccines, but also could be equally useful for postexposure treatment of filoviral infections.**

Lancet 2006; 367: 1399–404

Published Online
April 27, 2006
DOI:10.1016/S0140-6736(06)68546-2

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Introduction

Marburg virus (MARV) is a filovirus that causes a severe, and often fatal, haemorrhagic disease, for which there is currently no vaccine or therapy approved for human use. The reported potential of MARV as a biological weapon[1] and the recent attention drawn to outbreaks of emerging and re-emerging viruses, such as the 2004–05 epidemic of MARV haemorrhagic fever in Angola,[2] have greatly increased public recognition of this deadly pathogen.

The recent MARV outbreak in Angola, with case fatality rates approaching 90%, calls attention to the urgent need for effective countermeasures against filoviruses. So far, the only available form of treatment for MARV haemorrhagic fever is intensive supportive care. The development of effective treatments and therapies for the disease has been a continuing challenge since the disease was first recorded in 1967.[3] The requirement for biosafety level (BSL) 4 containment has been a major impediment towards the development of MARV therapeutics.

Guineapig and non-human primate models have been developed for MARV haemorrhagic fever.[4–11] Although these models have been used in several studies to investigate candidate vaccines, few studies have examined postexposure interventions. Several immunomodulatory drugs, including desferal, ridostin, and polyribonate, were investigated in guineapig models of experimental MARV infection; some protection and slight increases in mean time to death were recorded.[5,11] Despite the ability of several of these drugs to induce protective responses in guineapigs, the efficacy and action of these immunomodulators in non-human primates has yet to be determined. Furthermore, interferon has shown no substantial therapeutic potential against MARV infection in non-human primate models; similarly, ribavirin has shown no effect in guineapig models.[5,9,11]

Despite the slow progress in treatment development for MARV haemorrhagic fever, important advances have been made in the development of preventive vaccines against MARV and another filovirus, Ebola virus (EBOV). In particular, several recombinant vaccines have shown promising findings in non-human primate models of filoviral haemorrhagic fever, including vaccines based on recombinant adenoviruses[12,13] and recombinant alphaviruses.[8] We previously described the generation and assessment of a live, attenuated, recombinant vesicular stomatitis virus (rVSV) expressing the transmembrane glycoprotein of MARV (VSVΔG/MARVGP)[10,14] and showed that vaccination with this vector completely protected non-human primates against a lethal MARV challenge.[10] The rVSV vaccine platform

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
6 APR 2006	**N/A**	**-**

4. TITLE AND SUBTITLE
Postexposure protection against Marburg haemorrhagic fever with recombinant vesicular stomatitis virus vectors in non-human primates: an efficacy assessment, Lancet 367:1399 - 1404

5a. CONTRACT NUMBER
5b. GRANT NUMBER
5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER
5e. TASK NUMBER
5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)
11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
The original document contains color images.

14. ABSTRACT
BACKGROUND: Effective countermeasures are urgently needed to prevent and treat infections caused by highly pathogenic and biological threat agents such as Marburg virus (MARV). We aimed to test the efficacy of a replication-competent vaccine based on attenuated recombinant vesicular stomatitis virus (rVSV), as a postexposure treatment for MARV haemorrhagic fever. METHODS: We used a rhesus macaque model of MARV haemorrhagic fever that produced 100% lethality. We administered rVSV vectors expressing the MARV Musoke strain glycoprotein to five macaques 20-30 min after a high-dose lethal injection of homologous MARV. Three animals were MARV-positive controls and received non-specific rVSV vectors. We tested for viraemia, undertook analyses for haematology and serum biochemistry, and measured humoral and cellular immune responses. FINDINGS: All five rhesus monkeys that were treated with the rVSV MARV vectors as a postexposure treatment survived a high-dose lethal challenge of MARV for at least 80 days. None of these five animals developed clinical symptoms consistent with MARV haemorrhagic fever. All the control animals developed fulminant disease and succumbed to the MARV challenge by day 12. MARV disease in the controls was indicated by: high titres of MARV (10(3)-10(5) plaque-forming units per mL); development of leucocytosis with concurrent neutrophilia at end-stage disease; and possible damage to the liver, kidney, and pancreas. INTERPRETATION: Postexposure protection against MARV in non-human primates provides a paradigm for the treatment of MARV haemorrhagic fever. Indeed, these data suggest that rVSV-based filoviral vaccines might not only have potential as preventive vaccines, but also could be equally useful for postexposure treatment of filoviral infections.

15. SUBJECT TERMS **filovirus, Marburg, replication-competent vaccine, efficacy, laboratory animals, nonhuman primates**					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**SAR**	**6**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

shows potential as a preventive strategy against MARV infection; however, the efficacy of this system as a postexposure prophylaxis has yet to be determined.

The capacity of vaccines as postexposure treatments is shown by the management of several viral infections including rabies,[15,16] hepatitis B,[17] and smallpox.[18,19] With the use of postexposure vaccination to manage these viral diseases, and the success of our VSVΔG/MARVGP vector as a preventive one-shot vaccine,[10] we aimed to assess the therapeutic efficacy of a postexposure vaccination strategy using VSVΔG/MARVGP vectors in a non-human primate model of lethal MARV haemorrhagic fever.

Methods

Recombinant vectors and virus

The rVSV expressing the glycoproteins of MARV strain Musoke (MARV-Musoke) and Zaire EBOV (ZEBOV; strain Mayinga) were generated with the infectious clone for the VSV Indiana serotype.[14] Briefly, the appropriate open reading frames for the glycoproteins were generated by PCR, cloned into the VSV genomic vectors (without the VSV surface glycoprotein [G] gene), sequenced-confirmed, and rescued, as described elsewhere.[14,20] The recombinant viruses expressing MARV-Musoke and ZEBOV glycoproteins were referred to as VSVΔG/MARVGP and VSVΔG/ZEBOVGP. For the challenge studies, we used MARV-Musoke, which was isolated from a human case in 1980 in Kenya.[21]

Animal studies

We used eight healthy rhesus macaques (*Macaca mulatta*), aged 4–6 years and weighing 3–6 kg in this study. Animals were inoculated intramuscularly with 1000 plaque-forming units (pfu) of MARV-Musoke. About 20–30 min after MARV challenge, five (animals 1 to 5) of the eight animals were treated intramuscularly with a dose of 1×10^7 pfu of VSVΔG/MARVGP vectors that was divided among four different anatomical locations (right and left tricep, and right and left caudal thigh). The three remaining animals (animals 6 to 8) were controls and were treated with an equivalent dose of VSVΔG/ZEBOVGP vectors. We closely monitored animals for evidence of clinical symptoms. Blood samples were obtained before MARV challenge and on days 3, 6, 10, 14, 22, and 37 after challenge. Survivors were kept for more than 80 days. We did animal studies in a BSL-4 containment laboratory at the US Army Medical Research Institute of Infectious Diseases (USAMRIID) and approved by the USAMRIID Laboratory Animal Care and Use Committee. Animal research was undertaken in compliance with the Animal Welfare Act and other US federal statutes and regulations relating to animals and experiments on animals. Our animal work also adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility used was fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

For more information on the **Guide for the Care and Use of Laboratory Animals**, see http://www.nap.edu/readingroom/books/labrats/chaps.html/

Virus detection

We isolated RNA from blood using appropriate RNA isolation kits (QIAGEN, Mississauga, ON, Canada). To detect VSV, we used an RT-PCR assay targeting the matrix gene (nucleotide position 2355–2661, NC_001560).[10] MARV RNA was detected by use of primer pairs targeting the L polymerase gene.[10] The low detection limit for this MARV assay is 0·1 pfu/mL of plasma. We measured amounts of infectious MARV by plaque assay on Vero E6 cells from all blood samples.[22] Briefly, we adsorbed dilutions of plasma increasing ten-fold to Vero E6 monolayers in duplicate wells of a standard 6-well plate (0·2 mL/well); thus, the limit for detection of this plaque assay was 25 pfu/mL.

Haematology and serum biochemistry

Total white-blood-cell counts, red-blood-cell counts, platelet counts, haematocrit values, total haemoglobin, mean cell volume, mean corpuscular volume, and mean corpuscular haemoglobin concentration were measured from blood samples in tubes containing EDTA, by use of a laser-based haematological analyser (Coulter Electronics, Hialeah, FL, USA). The white-blood-cell differentials were done manually on Wright-stained blood smears. We tested serum samples for concentrations of albumin, amylase, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ glutamyltransferase, glucose, cholesterol, total protein, total bilirubin, urea nitrogen, and creatinine by using a Piccolo Point-Of-Care blood analyser (Abaxis, Sunnyvale, CA, USA).

Humoral immune response

We detected IgG and IgM antibodies against MARV with an ELISA, using purified viral particles as an antigen source.[10] Neutralisation assays were done by the measurement of plaque reduction in a constant virus-serum dilution format, as previously described.[10] Briefly, we incubated a standard amount of MARV (about 100 pfu) with serial dilutions (two-fold) of serum samples for 60 min. The mixture was then used to inoculate Vero E6 cells for 60 min. Cells were overlaid with an agar medium, incubated for 8 days, and plaques were counted 48 h after neutral red staining. Endpoint titres were measured by the dilution of serum that neutralised 50% of the plaques, with the plaque reduction neutralisation test ($PRNT_{50}$).

Cellular immune responses

The method for assessment of T-cell responses to MARV has been previously shown.[10] Briefly, peripheral blood mononuclear cells from rhesus macaques were isolated by histopaque gradient (Sigma, St Louis, MI, USA). About 1×10^6 cells were stimulated in 200 µL of RPMI media (Gibco, Invitrogen, Carlsbad, CA, USA) for 6 h at 37°C, with antibodies specific for CD28 and CD49d, brefeldin A, and with either dimethyl sulfoxide or a pool of 15-nucleotide coding sequences for peptides spanning the open reading frames for the gene encoding the

MARV-Musoke glycoprotein. The peptides were 15 aminoacids long, overlapping by 11, and were used at a final concentration of 2 µg/mL. Cells were fixed and made permeable with FACS lyse (Becton Dickinson, San Jose, CA, USA) supplemented with Tween 20, and then stained with a mixture of antibodies against CD3, CD4, CD8, and either tumour necrosis factor (TNF) α or interferon γ. Samples were run on a fluorescence-activated cell sorting analyser (FACS Calibur, Becton Dickinson) and analysed with the software FlowJo (version 7.0.5). Cytokine-positive cells were defined as a percentage in individual lymphocyte subsets, and at least 200 000 events were analysed for every sample.

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Three of the five animals challenged with MARV and subsequently treated with the VSVΔG/MARVGP vectors became febrile by day 6; however, body temperatures returned to prechallenge values by day 10. Importantly, all five animals survived the MARV challenge. By contrast, one of the three control animals (treated with non-specific VSVΔG/ZEBOVGP vectors) developed a fever at day 6 and the remaining two control animals became febrile by day 10. Disease progression in these controls was consistent with MARV infection in rhesus macaques. All the three control animals developed macular rashes by day 10 and succumbed to the MARV challenge, with two animals dying on day 11 and the remaining animal dying on day 12 (figure 1).

To determine whether viraemia of the rVSV vectors took place after treatment, whole blood samples from all eight treated animals were analysed by RT-PCR (data not shown). A transient rVSV viraemia was detected in four of the five VSVΔG/MARVGP-treated animals and two of the three control animals on day 3. We also analysed MARV replication from blood samples taken after MARV challenge and rVSV vector treatment (table 1). All the three control animals developed high MARV titres by day 6 (about 10^3 to 10^5 pfu/mL). By contrast, no MARV was detected in plasma by plaque assay at any timepoint from the five animals treated with the VSVΔG/MARVGP vectors after MARV challenge. However, RT-PCR showed a transient MARV viraemia at day 3 in four of the five specifically treated animals.

With respect to the analysis of blood chemistry and haematology, no substantial changes (greater than three-fold change compared with values before challenge) were detected in the five animals treated with the VSVΔG/MARVGP vectors during this study. However, the three control animals developed leucocytosis with concurrent neutrophilia at end-stage disease. Additionally, the three control animals showed substantial increases in circulating concentrations of alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, γ glutamyltransferase, and total bilirubin at day 10, suggesting severe damage to the liver. Two control animals also showed substantial increases in concentrations of urea nitrogen at day 10, and reduced concentrations of amylase at day 10, indicating possible injury of the kidneys and pancreas.

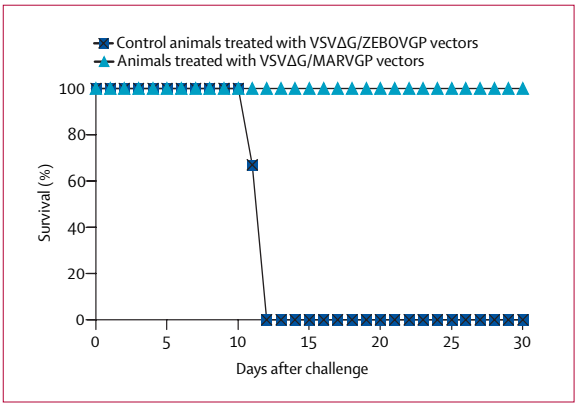

Figure 1: **Kaplan-Meier survival curves of rhesus monkeys treated with rVSV vectors after MARV challenge**

	VSV-MARV-treated animals					Control animals		
	1	2	3	4	5	6	7	8
Day 3	0	0	0	0	0	0	0	0
Day 6	0	0	0	0	0	3·2	3·7	5·5
Day 10	0	0	0	0	0	7·5	7·5	6·4
Day 11	0	0	0	0	0	7·0	7·4	6·9*

Viraemia measured as MARV titres $\log_{10}$ pfu/mL. Days indicate period after MARV challenge. *Measured on day 12.

Table 1: **Plasma viraemia of non-human primates after challenge with MARV and treatment with rVSV vectors**

As shown by their serological response profiles after treatment, all five animals treated with VSVΔG/MARVGP vectors showed low to moderate amounts of IgM (endpoint dilution titres 1:32 to 1:100) by day 6 (table 2); four of the five treated animals showed moderate amounts of IgG (≥1:100) by day 10 (table 2). Plaque reduction neutralisation tests showed low amounts of neutralising antibodies (1:10 to 1:80) from day 6 to day 37 in the plasma of all five animals treated with VSVΔG/MARVGP (figure 2).

To better understand how T lymphocytes mediate protection against MARV challenge, we used FACS analysis. Intracellular staining of fractions of peripheral blood mononuclear cells showed an absence of interferon γ and TNF α induction in all animals, suggesting an absence of T-lymphocyte activation (data not shown). Our inability to detect a cellular immune response is consistent with previous investigations of the VSVΔG/MARVGP vectors as a preventive vaccine.[10]

	VSV-MARV-treated animals					Control animals		
	1	2	3	4	5	6	7	8
IgM response profile								
Day 3	0	0	0	0	0	0	0	0
Day 6	32	100	100	32	32	0	0	0
Day 10	100	32	0	32	32	0	0	0
Day 14	0	32	0	0	100	..	..	..
IgG response profile								
Day 6	32	0	0	0	0	0	0	0
Day 10	1000	320	0	100	100	0	0	0
Day 14	1000	3200	32	320	100	..	..	..
Day 22	320	3200	32	320	1000	..	..	..
Day 37	320	320	100	320	1000	..	..	..

Data are endpoint dilution titres. Days indicate period after MARV challenge.

Table 2: **Serological response profiles of MARV infection after treatment with VSVΔG/MARVGP vectors**

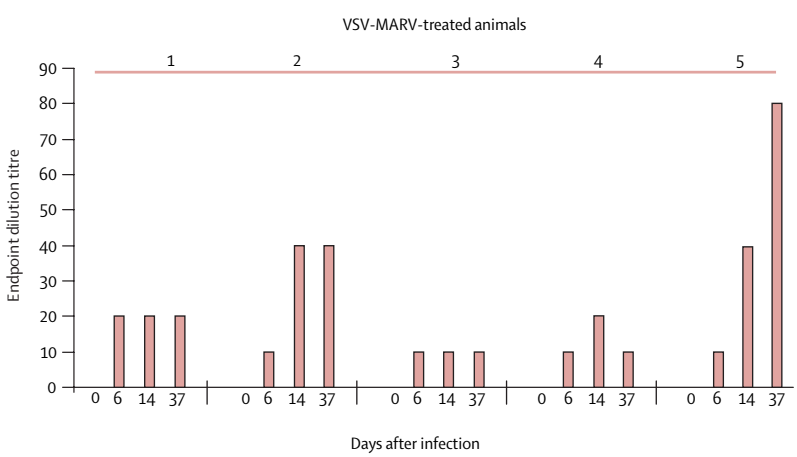

Figure 2: **Development of neutralising antibodies in animals treated with VSVΔG/MARVGP after MARV challenge**

Discussion

Use of the rVSV-based vector system as a countermeasure against MARV haemorrhagic fever shows dual efficacy, both as a potential preventive vaccine[10] and as a possible postexposure treatment. Here, we show that rVSV-based vectors expressing the glycoprotein of MARV can mediate postexposure protection against a homologous MARV challenge in non-human primates. The interval between MARV challenge and treatment was 20–30 min, which was chosen to represent a realistic amount of time that would be consistent with the treatment of an accidental needlestick exposure involving a laboratory or health-care worker. With two recent laboratory accidents with ZEBOV recorded[23,24] and with the increased construction of new BSL-4 laboratories worldwide, the probability for such occurrences will increase greatly in the next decade.

The experimental conditions that we used in this study represent a worse-case scenario of a needlestick exposure and a very high dose of infectious MARV (1000 pfu). In guineapig models of MARV haemorrhagic fever, 1000 pfu is more than 10 000 LD_{50} (unpublished data). We selected the MARV dose for our non-human primate studies on the basis of a potential accident involving exposure by injection of 0·1–0·2 µL blood from an infected animal or patient at peak viraemia (about 10^7 pfu/mL or 1000 pfu/0·1 µL of plasma). For both EBOV and MARV infections, lower challenge doses are known to delay the disease course in non-human primates by several days or more per 100-fold reduction in challenge dose.[13,25] Therefore, since many human filoviral exposures are probably less than 1000 pfu, and are transmitted by mucosal routes or abrasions rather than injection, the therapeutic window is probably longer than 20–30 min in these cases. This observation suggests that in addition to treating accidental exposures, rVSV-based treatment after exposure could also be used to treat individuals who have potentially been exposed to MARV-infected patients, since these patients would probably not have been exposed to such high-challenge doses as those used in our macaque model. Future studies should focus on determining how far after MARV challenge can treatment be successfully initiated in the non-human primate model; whether this treatment using rVSV vectors based on the Musoke strain of MARV can protect against other strains of MARV; and whether homologous vectors will be needed.

Although the mechanisms and correlates of protection against MARV remain to be determined, possible processes include interference or competition for target cells caused by the rVSV vectors. Noble and colleagues[26] described a non-infectious, defective interfering influenza A virus that disrupted the replication of a virulent influenza A virus when the viruses were administered simultaneously to mice. Furthermore, this interference prevented clinical disease in the mice. Our rVSV vectors, which exploit the MARV glycoprotein for binding and entry, could, in fact, interfere with MARV replication since they target the same host cells as wild-type MARV. Even an alteration or delay in the disease course could be enough to tip the balance in favour of the host. The fact the VSVΔG/ZEBOVGP vectors did not seem to have a delaying effect on the disease course in the control animals could indicate differences in cell-surface binding receptors between EBOV and MARV. In addition to interference, other possible protective mechanisms might include activation of the innate immune system or specific activation of cellular or humoral immune responses.

Our results suggest that the VSVΔG/MARVGP vectors induced protection, at least partly, through responses to the surface glycoprotein, presumably by stimulation of glycoprotein-specific antibodies. Specifically, low concentrations of neutralising antibodies and IgM were detected in serum samples 6 days after challenge, whereas increased amounts of anti-MARV IgG developed after 10–37 days. Although these data suggest that neutralising antibodies could participate in postexposure

protection, the contribution of non-neutralising antibodies and the therapeutic activity of antibody-mediated effector mechanisms probably had a more important role in protection. Indeed, recent studies of VSVΔG/MARVGP vectors as a preventative one-shot vaccine against MARV have suggested that protection is associated with humoral immune responses, notably by non-neutralising antibodies, since neutralising antibodies had been poorly induced.[10]

Many examples of preventive vaccine approaches link protective immune responses with non-neutralising antibodies. Monoclonal antibodies specific for the VSV G protein that had no neutralising activity against VSV in vitro were shown to completely protect mice against a lethal VSV challenge.[27] In this study, an intact Fc portion of a non-neutralising antibody was needed for in-vivo protection. Fc-mediated effector systems can induce lysis or clearance of virus-infected cells by antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity. In another study,[28] mice were vaccinated with a similar rVSV vector in which the VSV G gene was replaced by the fusion G gene of respiratory syncytial virus (RSV). The vaccine induced detectable serum antibodies against RSV by ELISA but no detectable neutralising antibodies, yet still protected the mice from RSV challenge.

The importance of the antibody response to the overall success of this VSV-based treatment is further supported by the absence of detectable cellular immune responses in the specifically treated animals. The absence of T-lymphocyte activation recorded in this study suggests that antibody production could occur partly through a T-lymphocyte-independent mechanism. Protective concentrations of antibodies have been recorded for several viral agents including VSV,[29] influenza,[30–32] and polyomavirus[33] in T-lymphocyte-deficient mice. T-lymphocyte-independent production of IgM could take place via strong crosslinking of the B-cell receptor. Antigens that can induce antibodies via a T-lymphocyte independent mechanism often consist of rigidly arranged, repetitive antigenic determinants that are spaced 5–10 nm apart. Previous work with VSV has suggested that particle-associated G protein, rather than soluble portions of the G protein or the G protein alone, is necessary for T-cell independent antibody induction.[34,35] Because our rVSV vectors are uncomplicated glycoprotein exchange vectors, it would not be surprising if the MARV glycoproteins were incorporated into the VSV virion structure in a similar way to authentic VSV glycoproteins; therefore, MARV glycoproteins in the background of the rVSV vector could also be capable of inducing antibody production via a T-cell-independent mechanism.

The protective mechanism of the VSVΔG/MARVGP vaccine in rhesus macaques as shown in this study remains to be determined. However, from a historical perspective, the mechanism for postexposure protection of humans against smallpox and rabies are also not fully understood. Clearly, more studies are needed to unravel the mechanisms by which VSVΔG/MARVGP vectors mediate protective immune responses as a postexposure treatment. However, these results evidently have important clinical implications, and offer a new treatment approach for MARV haemorrhagic fever and perhaps for other viral haemorrhagic fevers. Furthermore, these results suggest that single-shot vaccination regimens using these rVSV vectors as preventive vaccines (currently at 28 days between vaccination and filovirus challenge) can be substantially reduced.

Contributors

K M Daddario-DiCaprio and T W Geisbert contributed equally. K M Daddario-DiCaprio, T W Geisbert, S M Jones, and H Feldmann designed, planned, and coordinated the study; and wrote the manuscript. K M Daddario-DiCaprio, T W Geisbert, and J B Geisbert undertook the infection experiments, treated the animals, and obtained the study samples. K M Daddario-DiCaprio and J B Geisbert also did virological and immunological assays. A Grolla and L Fernando investigated the viraemia of the vectors and Marburg virus by RT-PCR. E A Fritz and L E Hensley measured the cellular responses of the animals used in the study. U Ströher, E Kagan, P B Jahrling, and L E Hensley participated in the design and the undertaking of the study, and interpretation of data. All the authors saw and approved the final version of the manuscript.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

We thank Denise Braun, Daryl Dick, Friederike Feldmann, Andrea Paille, and Carlton Rice for technical assistance and assistance with animal care; and Martin Ottolini for helpful suggestions with study design. The study was supported in part by a grant from the Canadian Institute of Health Research (CIHR–MOP–43921) awarded to HF and by the Medical Chemical/Biological Defense Research Program and Military Infectious Diseases Research Program, US Army Medical Research and Material Command (project numbers 02-4-4J-081 and 04-4-7J-012). The opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the US Army.

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