VIROLOGY **251,** 28–37 (1998)
ARTICLE NO. VY989367

Marburg Virus Vaccines Based upon Alphavirus Replicons Protect Guinea Pigs and Nonhuman Primates

Michael Hevey, Diane Negley, Peter Pushko, Jonathan Smith, and Alan Schmaljohn[1]

Virology Division, United States Army Medical Research Institute for Infectious Diseases, Fort Detrick, Frederick, Maryland 21702

Received July 1, 1998; accepted August 12, 1998

Marburg virus (MBGV), for which no vaccines or treatments currently exist, causes an acute hemorrhagic fever with a high mortality rate in humans. We previously showed that immunization with either killed MBGV or a glycoprotein (GP) subunit prevented lethal infection in guinea pigs. In the studies reported here, an RNA replicon, based upon Venezuelan equine encephalitis (VEE) virus, was used as a vaccine vector; the VEE structural genes were replaced by genes for MBGV GP, nucleoprotein (NP), VP40, VP35, VP30, or VP24. Guinea pigs were vaccinated with recombinant VEE replicons (packaged into VEE-like particles), inoculated with MBGV, and evaluated for viremia and survival. Results indicated that either GP or NP were protective antigens while VP35 afforded incomplete protection. As a more definitive test of vaccine efficacy, nonhuman primates (cynomolgus macaques) were inoculated with VEE replicons expressing MBGV GP and/or NP. Three monkeys received packaged control replicons (influenza HA); these died 9 or 10 days after challenge, with typical MBGV disease. MBGV NP afforded incomplete protection, sufficient to prevent death but not disease in two of three macaques. Three monkeys vaccinated with replicons which expressed MBGV GP, and three others vaccinated with both replicons that expressed GP or NP, remained aviremic and were completely protected from disease.

INTRODUCTION

Marburg virus (MBGV) was first recognized in 1967, when an outbreak of hemorrhagic fever in humans occurred in Germany and Yugoslavia after the importation of infected monkeys from Uganda (Martini and Siegert, 1971; Smith *et al.*, 1967). Thirty-one cases of MBGV hemorrhagic fever were identified that resulted in seven deaths. The filamentous morphology of the virus was later recognized to be characteristic, not only of additional MBGV isolates, but also of Ebola virus (EBOV) (Johnson *et al.*, 1977; Pattyn *et al.*, 1977; Smith *et al.*, 1982). MBGV and EBOV are now known to be distinctly different lineages in the family Filoviridae, within the viral order Mononegavirales (Kiley *et al.*, 1982; Feldmann and Klenk, 1996).

Few natural outbreaks of MBGV disease have been recognized, and all proved self-limiting with no more than two cycles of human-to-human transmission. However, the actual risks posed by MBGV to global health cannot be assessed because factors which restrict the virus to its unidentified ecological niche in eastern Africa, and those that limit its transmissibility, remain unknown (Feldmann and Klenk, 1996). Concern about MBGV is further heightened by its known stability and infectivity in aerosol form (Belanov *et al.*, 1996; Frolov and Gusev Iu, 1996). Thus laboratory research on MBGV is necessarily performed at the highest level of biocontainment. To minimize future risk, our primary interest has been the identification of appropriate antigens and vaccine strategies that can provide immunity to MBGV.

Early efforts to demonstrate the feasibility of vaccination against MBGV were only partially successful, as inoculation with Formalin-inactivated viruses only protected about half the experimental animals (guinea pigs or nonhuman primates) from fatal disease (Ignat'ev *et al.*, 1991, 1996). We recently demonstrated that the MBGV GP, cloned into a baculovirus vector and expressed as a soluble antigen to be administered in adjuvant, was sufficient to protect most but not all guinea pigs from lethal MBGV challenge (Hevey *et al.*, 1997). In addition, purified, ^{60}Co-irradiated virus, administered in adjuvant, completely protected guinea pigs from challenge with either of two different strains of MBGV, thus setting a standard for future, more practicable, vaccine candidates (Hevey *et al.*, 1997). Experiences with EBOV vaccines have been similar to those with MBGV, reinforcing the difficulties of classical approaches (Lupton *et al.*, 1980). Recent efforts to develop EBOV vaccines, using three distinctly different approaches (vaccinia recombinants, VEE replicon, and naked DNA) to achieve viral antigen expression in cells of vaccinated animals, showed that nucleoprotein (NP) as well as GP protected BALB/c mice (VanderZanden *et al.*, 1998), whereas protection of

[1] To whom correspondence and reprint requests should be addressed at Department of Viral Pathogenesis and Immunology, Virology Division, USAMRIID, Fort Detrick, Frederick, MD 21702. Fax: (301) 619-2290. E-mail: alan_schmaljohn@detrick.army.mil.

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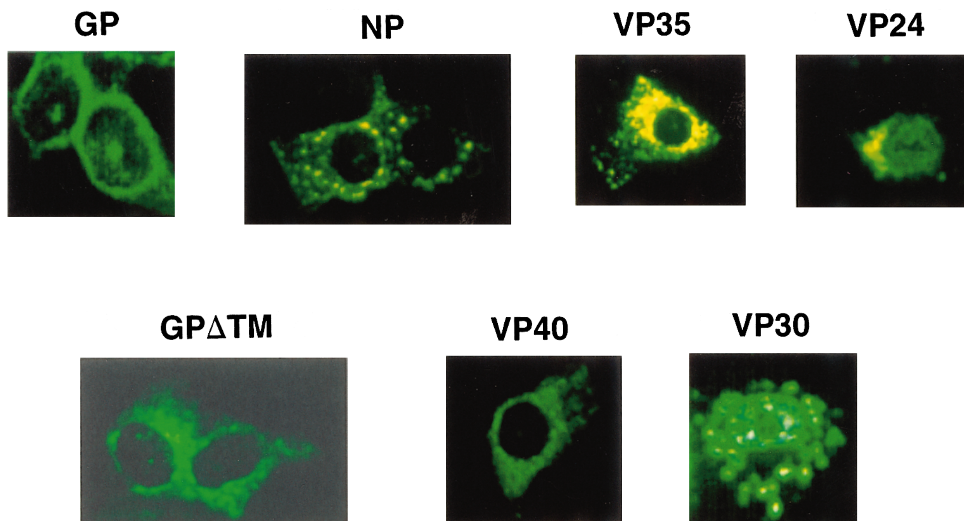

FIG. 1. Indirect immunofluorescence of Vero cells infected with packaged VEE replicons expressing the indicated antigens.

guinea pigs by NP was unsuccessful (Gilligan *et al.*, 1997, Pushko *et al.*, 1997a) or equivocal (Xu *et al.*, 1998).

Contemporary immunological wisdom predicts that protective responses to "internal" viral proteins, i.e., those inaccessible to antibodies (which, in the case of MBGV, presumptively includes all proteins other than GP), are best imparted by protein expression from within host cells. Therefore in this study a vaccine-delivery system based on a Venezuelan equine encephalitis (VEE) virus replicon was used to identify candidate protective antigens in addition to MBGV GP. In this vaccine strategy, a gene coding for a protein of interest is cloned in place of the VEE virus structural genes; the result is a self-replicating RNA molecule that encodes its own replicase and transcriptase functions and in addition makes abundant quantities of mRNA encoding the foreign protein. When replicon RNA is transfected into eukaryotic cells along with two helper RNAs that express the VEE structural proteins (glycoproteins and nucleocapsid), the replicon RNA is packaged into VEE virus-like particles by the VEE virus structural proteins, which are provided in *trans*. Since the helper RNAs lack packaging signals necessary for further propagation, the resulting VEE replicon particles (VRPs) that are produced are infectious for one cycle but are defective thereafter. Upon infection of an individual cell with a VRP, an abortive infection occurs in which the infected cell produces the protein of interest in abundance, is ultimately killed by the infection, but does not produce any viral progeny (Pushko *et al.*, 1997b).

Irrespective of how encouraging filovirus vaccine results may appear in guinea pigs or mice, protection of nonhuman primates is widely taken as the more definitive test of vaccine potential for humans. Low-passage viral isolates from fatal human cases of MBGV or EBOV tend to have uniform lethality in nonhuman primates but not in guinea pigs or mice. Small animal models with fatal disease outcomes have been achieved only with a subset of filovirus isolates and only then by multiple serial passages in the desired host (Hevey *et al.*, 1997; Bray *et al.*, 1998; Connolly *et al.*, 1998; Xu *et al.*, 1998). While highly useful for identification and initial characterization of vaccine candidates, guinea pig and murine models remain somewhat suspect with regard to the possibility that protection in such animals is easier to achieve than in nonhuman primates and, by inference, in humans. For example, with MBGV, peak viremias and viral titers in organs are more than 100 times higher in nonhuman primates than in guinea pigs. Results shown here demonstrate that the VEE replicon is a potent tool for vaccination with MBGV antigens. Guinea pigs were protected by vaccination with packaged replicons that expressed GP or by either of two replicons that expressed internal MBGV antigens (NP and VP35). GP expressed from the VEE replicon elicited an even more robust immunity than was achieved previously with a baculovirus-produced soluble GP administered in adjuvant. When results were extended to nonhuman primates, complete protection with GP was demonstrated. The data shown here constitute the most emphatic proof to date that an efficacious vaccine for MBGV is feasible and define candidate antigens for such a vaccine.

RESULTS

Analysis of protein products synthesized after infection of Vero cells with VEE replicons that expressed MBGV proteins

Results of indirect immunofluorescence assay (IFA) analyses of Vero cells infected with different recombinant VEE replicons expressing MBGV proteins are shown in Fig. 1. Expression of the indicated protein products was detected both with polyclonal guinea pig anti-MBGV and with monoclonal antibodies (MAbs) spe-

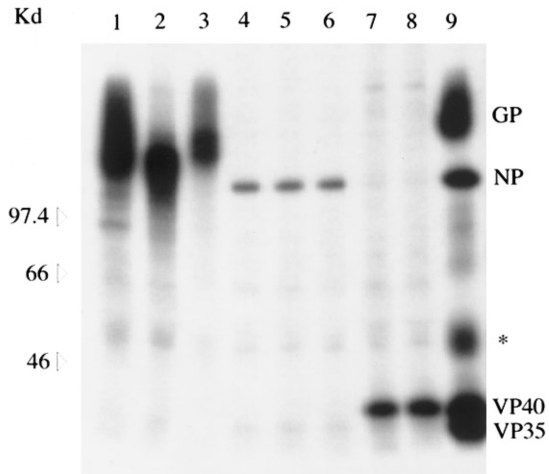

FIG. 2. Immunoprecipitation of MBGV proteins expressed from an alphavirus replicon in Vero cells using convalescent guinea pig polyclonal anti-MBGV serum. Lane 1, cell lysate from Vero cells infected with MBGV GP replicon; lane 2, cell lysate from Vero cells infected with MBGV GPΔTM replicon; lane 3, supernatant from Vero cells infected with MBGV GPΔTM replicon; lanes 4–6, cell lysate from Vero cells infected with various clones of MBGV NP replicon; lanes 7–8, cell lysate from Vero cells infected with various clones of MBGV VP40 replicon; lane 9, sucrose gradient-purified ^{35}S-labeled MBGV, *, an unidentified 46- to 50-kDa protein observed in virion preparations.

cific for the indicated MBGV proteins or, in the case of VP24 (for which no MAbs were available), with convalescent serum from a monkey that had survived infection with MBGV. There were distinct staining patterns for several of the expressed proteins. MBGV GP was observed as a plasma membrane fluorescence, while the GPΔTM provided a more diffuse cytoplasmic staining. These different staining patterns were not unexpected as GPΔTM, which lacks the hydrophobic transmembrane region of GP, is a secreted product. MBGV NP and VP35 formed discrete patterns in the cytoplasm of cells. MBGV VP40 demonstrated a more diffuse cytoplasmic staining pattern. MBGV VP30 was present in unique large globules staining in the cytoplasm of cells. MBGV VP24 staining was typically perinuclear. In summary, IFA served to assure that the appropriate antigen was expressed in a given preparation; it highlighted staining patterns, which demonstrated the localization of the expressed MBGV proteins in Vero cells; and it served as the basis for the assay whereby 10-fold dilutions of VRPs were quantitated for infectivity, as focus forming units (FFU).

Expression, antigenicity, and size determination of the MBGV proteins were confirmed by immunoprecipitation and gel electrophoresis. The results obtained from expression of MBGV GP, GPΔTM, NP, and VP40 in Vero cells are shown in Fig. 2. Products of the expected sizes were specifically immunoprecipitated from replicon-infected cell lysates. Glycosylation of MBGV GP more than doubles the predicted size of the peptide chain and typically results in a heterogeneous array of posttranslationally modified products (Feldmann *et al.*, 1991, 1994), especially in GP from cell lysates, as shown in Fig. 2, lane 1. As expected and shown previously in the baculovirus system, GPΔTM was secreted and thus present in the supernatant of replicon-infected cells (Fig. 2, lane 3). Appropriately, both the cell-associated (lane 2) and secreted (lane 3) forms of GPΔTM appeared smaller than the largest forms of GP (lane 1). The secreted form of GPΔTM appeared larger and somewhat more homogeneous than the same molecule from cell lysates, as noted previously (Hevey *et al.*, 1997) (compare Fig. 2, lanes 2 and 3). This difference likely reflects the more complete glycosylation of the secreted product compared to partially glycosylated forms of this protein expected to be present in the cell. In this gel, and with considerably less intensity in other preparations, an unidentified protein of approximately 46 kDa, which can be immunoprecipitated with GP-specific monoclonal antibodies (not shown), is evident in MBGV virions (Fig. 2, lane 9). Although it remains to be confirmed, this product may be the glycosylated form of a putative 27-kDa cleavage product of GP, reported to be the result of a posttranslational, furin-mediated cleavage of GP (Volchkov *et al.*, 1998). Replicon-expressed MBGV NP (Fig. 2, lanes 4–6) and VP40 (Fig. 2, lanes 7–8) comigrated with the authentic proteins present in purified MBGV virions. In other experiments, the reactivity with polyclonal or MAbs and the authentic electrophoretic migrations of the remaining replicon-expressed MBGV proteins (VP30, VP35, and VP24) were similarly demonstrated (data not shown).

Protective efficacy of VEE replicons expressing MBGV proteins in strain 13 guinea pigs

Groups of strain 13 guinea pigs were inoculated with packaged recombinant VEE replicons expressing individual MBGV proteins and later challenged with $10^{3.3}$ LD_{50} guinea pig-adapted MBGV subcutaneously. Results are shown in Table 1. MBGV GP protected guinea pigs from both death and viremia when administered as a three-dose regimen. In addition, no reduction in efficacy or potency was observed when a two-dose regimen was instituted, and significant efficacy was observed even when a single dose of 10^6 FFU of VRP-expressing MBGV GP was used as an immunogen. The efficacy of either the two- or three-dose vaccine schedule was further demonstrated by the observation that no boost in postchallenge ELISA titers were observed. This result suggested minimal antigen exposure after challenge with MBGV and thus robust or even sterile immunity in these animals. MBGV GPΔTM, which was previously shown to be protective as a vaccine when produced from insect cells, also protected guinea pigs from death and viremia when delivered in a VEE virus replicon. Again, there were no increases in postchallenge ELISA titers in the group of animals immunized with GPΔTM, thus no differences

TABLE 1

Protection of Replicon-Inoculated Strain 13 Guinea Pigs from Lethal Challenge with Marburg Virus (Musoke Isolate)[a]

Antigen	No. of doses replicon[c]	S/T[d]	Log_{10} ELISA titer[b] Day 7	Day 64	Viremia[e]	V/T[f]	MDD[g]
GP	3	6/6**	4.21	3.80	<1.7	0/6	—
GP	2	6/6**	4.30	4.06	<1.7	0/6	—
GP	1	5/6*	2.89	4.19	4.1	1/6	9
NP	3	6/6**	3.38	3.94	<1.7	0/6	—
VP40	3	1/6	2.83	2.68	4.5	5/6	10
GPΔTM	3	6/6**	3.93	3.65	<1.7	0/6	—
VP35	3	5/6*	1.99	3.75	3.7	5/6	13
VP30	3	0/6	2.23	—	5.8	6/6	10
VP24	3	1/6	<1.5	4.31	5.6	6/6	11
Lassa NP	3	1/6	<1.5	4.19	6.0	5/6	10
None	—	1/6	<1.5	4.25	5.2	5/6	11

[a] Guinea pigs were challenged with 3 Log_{10} PFU guinea pig adapted MBGV (Musoke) subcutaneously.
[b] Endpoint titer of equal volumes of serum pooled from animals in each group against MBGV Musoke.
[c] Each replicon doses consisted of 1×10^6 FFU.
[d] Survivors/Total (S/T) on Day 30 postinfection. $^{**}P < 0.01$, $^{*}P < 0.05$.
[e] Viremia (Log_{10} PFU/ml) Day 7 postinfection. Where ≥2 animals were viremic, a GMT was calculated.
[f] Viremic animals/total (V/T) on Day 7 postinfection. All animals that died were viremic.
[g] Mean day of death.

were discerned in the vaccine efficacy of membrane-bound versus soluble GP.

In the experiment shown, MBGV NP protected all vaccinated guinea pigs from both viremia and death, while MBGV VP35 vaccination resulted in five of six animals surviving, but four of the five survivors were viremic 7 days postinfection. None of the other MBGV viral proteins cloned into VEE replicons evoked significant protection against a lethal challenge with MBGV. Thus the proteins that showed the most promise as vaccine candidates in the guinea pig model were MBGV GP and NP. Cumulative results from this and additional experiments (not shown) in strain 13 guinea pigs inoculated three times with VRPs demonstrated complete survival with GP (18/18) and less complete protection with NP (16/18) and VP35 (13/18) as compared with controls (2/24).

Protection of cynomolgus monkeys vaccinated with recombinant VEE replicons expressing either MBGV GP and/or NP

Encouraged by the success in vaccinating guinea pigs against MBGV, we evaluated the ability of these same VEE replicons to protect cynomolgus macaques from lethal MBGV infection. The monkeys received 10-fold higher doses of replicons but on an identical schedule as tested in the guinea pigs. Four groups contained three monkeys each. One group received VRPs that expressed MBGV GP; a second group received VRPs that expressed MBGV NP; a third group received a mixture of MBGV GP and MBGV NP VRPs; and a fourth received VRPs that expressed a control antigen (influenza HA) irrelevant to MBGV immunity. Anti-MBGV ELISA antibody titers were monitored throughout the experiment.

All animals that received VEE replicons expressing MBGV GP, either alone or in combination with MBGV NP, survived challenge with 8000 PFU MBGV without any observed signs of illness (Table 2). Of the three animals vaccinated with MBGV NP, one died 8 days after challenge from MBGV disease. The other two NP recipients displayed signs of illness 7–9 days after challenge but eventually recovered. One NP-inoculated survivor had a relatively mild disease (slightly reduced activity and responsiveness), while the other had severe disease, which included obvious petechiae, loss of weight, reduced activity, and fever. All control animals succumbed, with clinical signs first noted on Day 7 or 8, and deaths occurring on Days 9 or 10 postchallenge.

TABLE 2

Protection of Replicon-Inoculated Cynomolgus Macaques from Lethal Challenge with Marburg Virus (Musoke Isolate)[a]

Replicon[b]	Survival/total	Sick/total[c]	Day of death
GP	3/3*	0/3	—
NP	2/3	3/3	8
GP + NP	3/3*	0/3	—
Influenza HA	0/3	3/3	9, 9, 10

[a] Monkeys were challenged with 8000 PFU MBGV (Musoke) subcutanously. Surviving animals remain healthy >90 days postchallenge. $^{*}P = 0.05$.
[b] Antigen delivered by VEE replicon.
[c] All animals that displayed signs of illness became viremic.

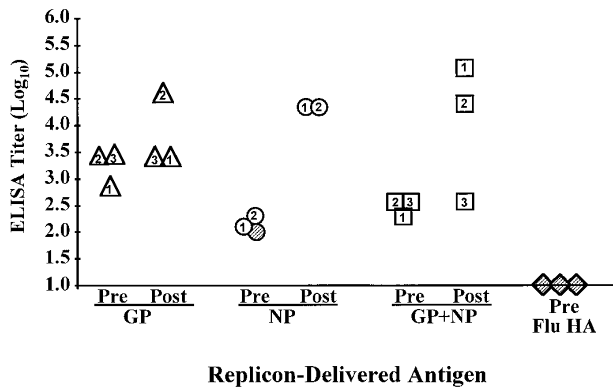

FIG. 3. Anti-MBGV ELISA titers of cynomolgus monkeys after three inoculations with recombinant replicon 17 days before or after challenge with MBGV. Prechallenge samples were obtained 17 days before challenge, while postchallenge samples were obtained 17 days after challenge. GP, animals inoculated with VEE replicons expressing MBGV GP; NP, animals inoculated with VEE replicon expressing MBGV NP; GP+NP, animals inoculated with a mixture of VEE replicons expressing either MBGV GP or NP; Flu HA, animals inoculated with VEE replicon expressing influenza HA. Numbers inside each symbol represent the same individual in each group. Symbols filled in with cross-hatch marks signify animals that died from infection.

The pre- and postchallenge ELISA antibody titers of the cynomolgus macaques are shown in Fig. 3. All animals inoculated with replicons that expressed MBGV proteins demonstrated prechallenge ELISA titers to purified MBGV antigen. Of the three GP-vaccinated animals that survived challenge, two demonstrated a modest boost in ELISA antibody titer (10- to 30-fold) when pre- and postchallenge samples were compared. The two surviving NP-inoculated macaques had larger boosts in ELISA antibody titers (100- to 300-fold) when pre- and postchallenge samples were compared. Two of three animals vaccinated with both GP and NP also demonstrated 100- to 300-fold rise in ELISA titers. These observations, in conjunction with the back titration of the MBGV challenge inoculum (8000 PFU), confirmed that all groups were unambiguously challenged, and that two monkeys had particularly robust immunity that apparently restricted virus replication below an immunogenic threshold.

A plaque reduction neutralization assay was performed on pre- and postchallenge serum samples. No neutralization activity was observed, at 1:20 or higher dilutions, in any sample. It should be noted that it is frequently difficult to demonstrate filovirus-neutralizing antibody *in vitro;* however, antibodies may nonetheless be relevant *in vivo* (Hevey *et al.,* 1998), perhaps via mechanisms other than classical neutralization (Schmaljohn *et al.,* 1982).

The viremia levels in each of the monkeys at several time points after MBGV challenge are shown in Fig. 4. The data illustrate the profound differences between lethally infected control animals and healthy survivors. Most striking, none of the animals vaccinated with GP, either alone or in combination with NP, had infectious MBGV virus in their sera that was detectable by plaque assay. Animals vaccinated with a replicon expressing influenza HA were all viremic by Day 3 postchallenge and demonstrated sharp rises in MBGV viremia levels which peaked at 7.5–8.0 Log_{10} PFU/ml on Day 7 postinfection. Among monkeys vaccinated with NP, one died with viremias indistinguishable from controls. In contrast, the two NP-vaccinated monkeys that recovered had peak viremias that were diminished ≥1000-fold compared with controls. By Day 10 postinfection, the NP-vaccinated monkey with the milder illness had no detectable viremia, while the more severely affected monkey still had ~4.5 Log_{10} PFU/ml virus. By Day 17 postinfection, no viremia was detectable in either of the surviving NP-vaccinated animals.

Additional measures of vaccine-mediated protection

Upon necropsy of the control and the unprotected NP-inoculated monkeys, MBGV titers in their livers were 9.2, 9.7, 9.4, and 9.6 Log_{10} PFU/g. Virus was detected in all other organs examined as well and although abundant, was at least 10-fold lower than in the liver. Not surprisingly, elevated liver enzymes were the most obvious abnormal feature in clinical chemistries. As shown in Fig. 5, unprotected monkeys had elevated AST levels by Day 5 or 7 postinfection, and these were paralleled by similarly profound increases in ALT and ALP (not shown). Terminal samples were automatically rejected by the instrument as too lipemic or hemolyzed; however, in a previous set of control monkeys, liver enzymes had continued to ascend dramatically (not shown). With regard to

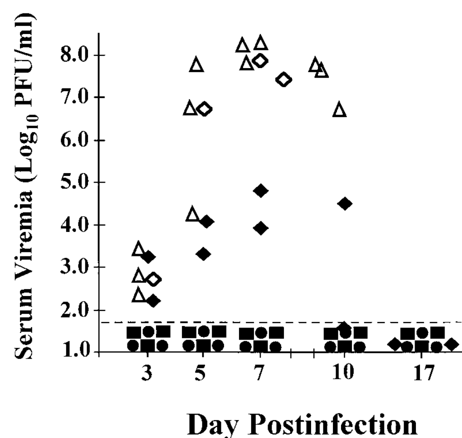

FIG. 4. Viremia level in cynomolgus monkeys inoculated with alphavirus replicons followed by challenge with MBGV (Musoke). ●, animals vaccinated with VEE replicons expressing MBGV GP; ◆, animals vaccinated with VEE replicons expressing MBGV NP; ■, animals vaccinated with a mixture of VEE replicons which expressed either MBGV GP or NP; △, animals vaccinated with VEE replicons expressing influenza HA. Open symbols represent animals that died. Closed symbols represent animals that lived. Dotted line notes the lower limit of detection of this plaque assay (1.7Log_{10} PFU/ml).

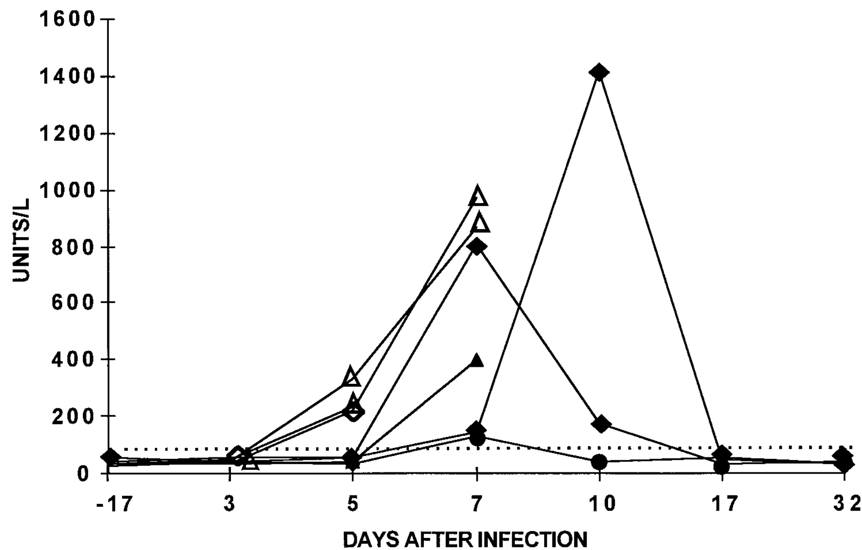

FIG. 5. Serum AST levels in VEE replicon inoculated cynomolgus macaques after challenge with MBGV (Musoke). ●, the one animal (of six) vaccinated with VEE replicons expressing MBGV GP that exhibited AST abnormality at any time point. ◆, animals vaccinated with VEE replicons expressing MBGV NP; △, animals vaccinated with VEE replicon expressing influenza HA. Open symbols represent animals that died. Closed symbols represent animals that lived. Dotted line demarks 88 U/L, which is the mean (38 U/L) plus three standard deviations of prebleed values from the 12 monkeys in this experiment.

vaccine-mediated protection, it is instructive that the two NP-inoculated survivors exhibited marked but transient rises in their liver enzymes (Fig. 5), which is consistent with their viremias and signs of MBGV disease. Also, the more severely affected NP-inoculated survivor exhibited a transient rise in urea nitrogen and creatinine (not shown), coincident with recovery and viral clearance. This may have been due to virus-antibody complexes perturbing kidney function or to direct viral damage to the organs. In contrast, the six monkeys vaccinated with GP exhibited either a minimal rise at one time point (i.e., the one GP animal shown in Fig. 5) or no significant increases in liver enzymes at any time evaluated. Other clinical chemistries and hematological findings remained normal in MBGV-inoculated macaques vaccinated previously with GP or GP+NP, in contrast with control monkeys that exhibited the expected profound end-stage abnormalities in both hematological and chemistry measurements (Johnson *et al.,* 1995).

DISCUSSION

To our knowledge, this is the first report of any filovirus vaccine shown to be completely efficacious in nonhuman primates. Before these observations, we were cautiously optimistic about the overall feasibility of an efficacious vaccine for MBGV but were also concerned that proofs of filovirus vaccine concepts in guinea pigs may not necessarily forecast success in nonhuman primates and, by inference, in humans. Results presented here defined GP, possibly in combination with NP, as candidate antigens for a MBGV vaccine and demonstrated that nearly complete immunity is achievable in nonhuman primates.

We chose an alphavirus replicon based on VEE virus to deliver the antigens of interest. This method of vaccination has several advantages (Pushko *et al.,* 1997b), including the ability to produce large quantities of antigen *in situ,* so that native processing of the antigens might evoke a broad array of immune responses. In addition, all transcription of RNA occurs in the cytoplasm of cells, which avoids RNA splicing problems sometimes observed when proteins of RNA viruses are expressed from the nucleus. Moreover, VEE replicons have proven stable after packaging into VRPs. In addition to robust antibody induction, alphavirus replicons have been demonstrated to elicit cytotoxic T lymphocytes in mice (Zhou *et al.,* 1995; Caley *et al.,* 1997). The success reported here using VEE replicons to vaccinate monkeys against lethal MBGV challenge justifies a more detailed analysis of the potential of these vectors for use as human vaccines. These analyses may include such factors as the relevance of host-vector interactions that may affect vaccine potency, overall safety of the vector, and the duration and minimal requirements for immunity to MBGV disease induced by this vector.

Two viral antigens demonstrated unambiguous potential as protective antigens in the guinea pig model: MBGV GP and MBGV NP. Another viral antigen, VP35, provided significant protection from death; however, most (5/6) animals vaccinated with VP35 exhibited viremias 7 days after infection. Consequently, VP35 was not considered a candidate for the initial examination of vaccine efficacy in nonhuman primates.

While none of the other viral antigens showed significant promise as protective antigens in the guinea pig model, some were only weakly immunogenic, at least when delivered as VRPs. Thus we have not formally excluded the possibility that such antigens may prove protective under different circumstances or in species other than guinea pigs.

As a more definitive test of efficacy, the two most promising guinea pig protective antigens from MBGV were used to inoculate nonhuman primates either alone or in combination. Using recombinant VEE replicons, MBGV GP was clearly shown to be protective. The observation that none of the animals developed overt illness or viremia was conclusive proof that this vaccine approach had protected animals from a substantial challenge dose of MBGV. However, there were some significant differences observed between guinea pigs and cynomolgus macaques. Most notable was the observation that two-thirds of the GP-vaccinated monkeys demonstrated rises in ELISA antibody titers following MBGV challenge, whereas there was apparently sterile immunity (i.e., no further increases in antibody titers) to viral challenge in guinea pigs given a 10-fold lower dose of the same vaccine. This may be attributable to the overall higher prechallenge ELISA antibody titers observed in guinea pigs when compared to those observed in the monkeys (Table 1 *vs* Fig. 3).

The second antigen examined, MBGV NP, was less effective at protecting nonhuman primates compared to guinea pigs. All the monkeys inoculated with NP displayed signs of illness, with one animal dying in the same time frame as control animals. All animals were viremic, and viremia levels were predictive of outcome. As expected, the two animals that survived illness had large boosts in their ELISA antibody titers against MBGV when pre- and postchallenge sera were examined. Though not statistically significant in a group of only three animals, MBGV NP was apparently able to provide a measure of protection from death but not from disease in two monkeys. We surmise that the immune response to NP was sufficient to suppress replication of MBGV until augmented by additional host immune responses.

The monkeys that were vaccinated with both MBGV GP and NP demonstrated the same degree of protection as the animals vaccinated with GP alone. No viremias were observed at any time point, and two of three animals demonstrated postchallenge increases in ELISA antibody titers to MBGV. These results demonstrated that the NP replicon, equivocal by itself as a macaque vaccine, did not interfere with a GP-based vaccine when protective efficacy was used as a measurement.

For these studies, in the interest of expedient vaccine development, protection from viral disease was prioritized over the detailed study of immune mechanisms in two relatively difficult animal species for immunological studies, guinea pigs and cynomolgus macaques. It was already clear from studies done in guinea pigs that ELISA antibody titers to MBGV were not wholly predictive of clinical outcome but rather one measure of immunogenicity of the vaccine candidate. However, it was also known that administration of polyclonal antisera or a neutralizing MAb could protect some guinea pigs from lethal challenge, indicating that antibodies can play a role in the protective response to MBGV (Hevey *et al.*, 1997). As for immunity to virtually all viruses, T cell responses to MBGV are almost certainly important in their immunoregulatory and effector functions. Indeed, we observed protection in both guinea pigs (NP and VP35) and nonhuman primates (NP) with antigens for which the most logical protective mechanisms involve cellular immunity. However, it also proved emphatically true in the most susceptible animals—nonhuman primates—that protective immunity was elicited by an antigen (GP) that theoretically favored a redundant protective response of both T cells and antibodies. For development and licensure of vaccines for human use, it will be necessary to understand the protective immune responses of nonhuman primates to MBGV and determine which responses (or which combinations of them) are most predictive of vaccine efficacy.

MATERIALS AND METHODS

Cell cultures and viruses

Vero E6 (Vero C1008, ATCC CRL 1586), Vero 76 (ATCC CRL 1587), and BHK (ATCC CCL 10) cells were grown in minimal essential medium with Earle's salts supplemented with 10% fetal bovine serum and gentamicin (50 μg/ml). MBGV (strain Musoke) was isolated from a human case in 1980 in Kenya (Smith *et al.*, 1982), and a derivative of this virus (six passages in Vero 76 cells) was used to challenge the cynomolgus monkeys. The MBGV (Musoke) that was adapted for guinea pig lethality and plaque-picked three times was described previously (Hevey *et al.*, 1997).

Construction of recombinant VEE replicons

MBGV gene clones pGem-GP, pGem-NP, pTM1-VP40, pTM1-VP35, pTM1-VP30, and pTM1-VP24 were generously provided by Heinz Feldmann and Anthony Sanchez (Centers for Disease Control and Prevention, Atlanta, GA). VEE replicon and shuttle vector as well as the replicons that express Lassa virus NP and Flu HA were previously described (Pushko *et al.*, 1997b). The MBGV GP gene from pGem-GP was excised with *Sal*I and subcloned into the *Sal*I site of the shuttle vector by using standard techniques (Sambrook *et al.*, 1989). A clone with the MBGV GP gene in the correct orientation was excised with *Apa*I and *Not*I, and this fragment was cloned into the *Apa*I and *Not*I sites of the VEE replicon plasmid.

Construction of pBluescript-KS(+)-GPΔTM, a deletion mutant of MBGV from which the C-terminal 39 amino acids (transmembrane region and cytoplasmic tail) of MBGV GP were removed, was previously described (Hevey *et al.,* 1997). Here, the MBGV GPΔTM gene was excised from pBluescript-KS(+) with *Hin*dIII, and the resulting fragment ligated into the *Hin*dIII site of the shuttle vector. MBGV GPΔTM gene was excised from the shuttle vector using *Cla*I, and the resulting fragment ligated into the VEE replicon plasmid.

The MBGV NP gene was amplified by PCR performed with 1 ng of pGem NP as template DNA, 1 μg each of forward (5′-CCG ACC ATG GAT TTA CAC AGT TTG TTG G-3′) and reverse primer (5′-CTA GCC ATG GCT GGA CTA CAA GTT CAT CGC-3′), and AmpliTaq polymerase (GeneAmp PCR reagent kit, Perkin-Elmer, Branchburg, NJ). The reaction conditions were: 40 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min, followed by a final extension step at 72°C for 5 min. The product was cloned into the pCRII vector, excized with *Eco*RI, then subcloned into the shuttle vector using *Eco*RI sites. The MBGV NP gene was excised with *Cla*I and ligated into the VEE replicon plasmid.

The MBGV VP40, VP35, VP30, and VP24 genes were excised from pTM1 with *Bam*HI and ligated into the *Bam*HI site of the shuttle vector. These MBGV genes were then excised from shuttle vectors using either *Cla*I (VP35, VP30, and VP24) or *Apa*I and *Not*I (VP40) and ligated into the VEE replicon plasmid.

Packaging of replicons into VEE-like particles and determination of replicon titer

Replicon RNAs were packaged into VRPs as described previously (Pushko *et al.,* 1997b). Briefly, BHK cells were cotransfected with RNA transcribed *in vitro* from the replicon plasmid and from two helper plasmids, one of which expressed VEE glycoproteins and the other VEE capsid protein. The cell culture supernatant was harvested approximately 30 h after transfection, and the replicon particles were concentrated and partially purified by pelleting through a 20% sucrose cushion (SW28 rotor, 25,000 rpm, 4 h), after which they were resuspending in 1 ml PBS. To assay titers of packaged replicons, Vero cells (10^5 cells per well in eight-chamber slides, Labtek slides, Nunc Inc.) were infected with serial dilutions of the replicon particles and incubated for 16–18 h at 37°C to allow for expression of the MBGV genes. After rinsing and fixating with acetone, antigen-positive cells were identified by indirect immunofluorescence assay (IFA) as described previously (Schmaljohn *et al.,* 1995). The antibodies used included MAbs specific for MBGV GP (II-7C11), NP (III-5F8), VP40 (III-1H11), VP35 (XBC04-BG06), and VP30 (III-5F11 and 5F12) (Hevey *et al.,* 1997). To detect VP24 antigen, a monkey anti-MBGV serum was used, a monkey anti-Lassa serum was used to detect expression of Lassa NP in cells, and influenza HA was detected with serum from a mouse immunized with a VEE replicon expressing influenza HA (provided by Dr. Mary Kate Hart, USAMRIID).

Immunoprecipitation and gel electrophoresis of proteins expressed by VEE replicons

Expressed MBGV antigens were immunoprecipitated and analyzed by gel electrophoresis as described previously (Hevey *et al.,* 1997). Briefly, Vero cells were infected (m.o.i. ≥3) with VRP expressing a single MBGV antigen. Complete medium was replaced 16–18 h postinfection by methionine- and cysteine-free medium for 1 h, and monolayers were then labeled with ^{35}[S]methionine and cysteine for 4 h. Convalescent guinea pig anti-MBGV (Group 1, Table 5, in Hevey *et al.,* 1997) was used to immunoprecipitate MBGV-specific proteins from the resulting cell lysates.

Vaccination of guinea pigs with VEE replicons expressing MBGV proteins

Inbred strain 13 guinea pigs (maintained as a colony at USAMRIID) were inoculated subcutaneously with 10^6 focus-forming units (FFU) of VRP in a total volume of 0.5 ml administered at two dorsal sites. Guinea pigs were anesthetized and bled, and those that received two or three doses of replicon were inoculated (as described for the first vaccine dose) 28 days after the primary vaccination. Guinea pigs were anesthetized and bled again 28 days later, and animals that received three doses of replicons were inoculated, as described above. Animals were anesthetized and bled 21 days later, and challenged 7 days after the last bleed with $10^{3.0}$ plaque forming units (PFU) (ca. 2000 LD_{50}) guinea-pig-adapted MBGV. Animals were examined daily for signs of illness. Heparinized plasma was obtained from the retroorbital sinus of anesthetized animals 7 days postinfection for assay of viremia. Surviving guinea pigs were observed for at least 30 days after challenge, then anesthetized, and exsanguinated. Viremia titers were measured by plaque assay on Vero E6 cells.

Vaccination of cynomolgus monkeys with replicons

Twelve cynomolgus macaques (*Macaca fascicularis*), 11 females and 1 male, ranging from 2.8 to 4.5 kg, were inoculated subcutaneously with 10^7 FFU of VRP in a total volume of 0.5 ml at one site. Monkeys were anesthetized with ketamine, bled, and inoculated (as described for the first vaccine dose) 28 days after the primary injection, and again 28 days after the second. Animals were anesthetized and bled 21 days after the third vaccine dose, then were challenged 14 days later with $10^{3.9}$ PFU MBGV subcutaneously. Here and in guinea pig experiments, the inoculum was back-titrated to ensure proper dose delivery. Animals were examined daily by the attending vet-

erinarian for signs of illness and given buprenorphine (Buprenex) at a dosage of 0.01 mg/kg body weight, BID, to provide analgesia upon signs of distress. Of the unprotected animals, three succumbed abruptly, while one was euthanized in extremis. A detailed clinical evaluation, serum for viremia determination and blood chemistries, as well as EDTA blood was obtained from anesthetized animals 17 days before and 3, 5, 7, 10, 17, and 32 days postinfection. Viremia was measured by plaque assay on Vero E6 cells.

MBGV ELISA and infectivity assays

Antibody titers in guinea pig plasmas or monkey sera were determined by an indirect ELISA as described previously (Hevey *et al.,* 1997). Briefly, antigen consisting of purified, irradiated virus was coated directly onto PVC plates and serial dilutions of test serum were added to wells containing antigen. The presence of bound antibody was detected by use of the appropriate horseradish peroxidase conjugated anti-species antibody (HPO-goat-anti-guinea pig IgG H+L; HPO-goat-anti-monkey IgG H+L). Endpoint of reactivity was defined as the dilution at which OD_{405} was 0.2 as determined by extrapolation of a four parameter curve fit (SOFTmax, Molecular Devices Corp.) of background-subtracted mean OD versus dilution. Results shown in any table or figure are from a single assay to allow more valid comparison of endpoints. Plaque assays were performed on Vero E6 cells with a semisolid overlay on serial dilutions of samples. Viral plaques were visualized by staining viable cells with Neutral red 6–7 days postinfection. To measure plaque reduction neutralization, equal volumes of a virus stock (target plaque dose was 100 PFU) and serum diluted in cell culture medium were mixed and incubated at 37°C for 1 h. The resulting sample was assayed by plaque assay on Vero E6 cells for more than a 50% reduction in PFU compared to control samples.

Clinical laboratory assays

For nonhuman primate studies, hematological results were obtained with a Coulter instrument, and differential counts were performed manually. Clinical chemistry results were obtained with a Piccolo analyzer (Abaxis, Inc.) using the diagnostic panel General Chemistry 12, which measures alanine aminotransferase (ALT), albunin, alkaline phosphatase (ALP), amylase, aspartate aminotransferase (AST), calcium, cholesterol, creatinine, glucose, total bilirubin, total protein, and urea nitrogen.

ACKNOWLEDGMENTS

The views, opinions and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation. In conducting research using animals, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH publication No. 86–23, revised 1996). We acknowledge and highly value the professional assistance of Joan Geisbert, Merhl Gibson, Peter Schultheiss, Peter Vogel, Neal Woollen, and Nancy Jaax, especially with regard to inherently hazardous BL4 animal experiments, Steve Hatfill for BL4 clinical chemistry assays, Paul Gibbs for statistical analyses, and Peter Jahrling for helpful critical insight. The authors thank Anthony Sanchez from the Centers for Disease Control and Prevention, Atlanta, GA, and Heinz Feldmann from the Institut für Virologie, Philipps-Universität, Marburg, Germany, for graciously providing the cloned MBGV (Musoke) genes.

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