

# Marburg Virus Angola Infection of Rhesus Macaques: Pathogenesis and Treatment with Recombinant Nematode Anticoagulant Protein c2

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**Background.** The procoagulant tissue factor (TF) is thought to play a role in the coagulation disorders that characterize filoviral infections. In this study, we evaluated the pathogenesis of lethal infection with the Angola strain of Marburg virus (MARV-Ang) in rhesus macaques and tested the efficacy of recombinant nematode anticoagulant protein c2 (rNAPc2), an inhibitor of TF/factor VIIa, as a potential treatment.

**Methods.** Twelve rhesus macaques were challenged with a high dose (1000 pfu) of MARV-Ang. Six macaques were treated with rNAPc2, and 6 macaques served as control animals.

**Results.** All 6 control animals succumbed to MARV-Ang challenge by day 8 (mean, 7.3 days), whereas 5 of 6 rNAPc2-treated animals died on day 9 and 1 rNAPc2-treated animal survived. The disease course for MARV-Ang infection appeared to progress more rapidly in rhesus macaques than has been previously reported for other strains of MARV. In contrast to Ebola virus (EBOV) infection in macaques, up-regulation of TF was not as striking, and deposition of fibrin was a less prominent pathologic feature of disease in these animals.

**Conclusions.** These data show that the pathogenicity of MARV-Ang infection appears to be consistent with the apparent increased human virulence attributed to this strain. The apparent reduced efficacy of rNAPc2 against MARV-Ang infection, compared with its efficacy against EBOV infection, appears to be associated with differences in TF induction and fibrin deposition.

The family *Filoviridae* contains two genera, *Ebolavirus* and *Marburgvirus* [1]. Although the Ebola virus (EBOV) genus includes 4 distinct species, the Marburg virus (MARV) genus consists of a single species, *Lake Victoria marburgvirus*. EBOV has been associated with larger outbreaks and higher case-fatality rates than has MARV, with mortality rates for the Zaire species of EBOV approaching 90% in some settings. The initial outbreak of MARV infection in Europe in 1967, which was traced to nonhuman primates imported from Uganda, resulted in 7 deaths among a cohort of 32 confirmed case patients [2, 3]. Three isolated episodes

of Marburg hemorrhagic fever (MHF) in Africa between 1975 and 1987 resulted in 3 deaths among 6 total case patients [4–6]. These occurrences between 1967 and 1987 suggested that MARV may be less pathogenic than EBOV. However, a large outbreak of MHF in northeastern Democratic Republic of the Congo between October 1998 and September 2000 offered the first hint that MARV may be just as lethal in primates as its more famous cousin, EBOV [7]. In that outbreak,

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48 of 154 cases of infection were laboratory confirmed, and 106 were suspected. Among the 48 laboratory-confirmed cases, the case-fatality rate was 56%, and the mortality rate was ~83% when all cases, both laboratory confirmed and suspected, were considered. Although previous episodes of MHF each involved a single distinct strain of MARV, this outbreak involved at least 9 genetically distinct strains of MARV [7].

MARV resurfaced in eastern Africa in 1998–2000, after an absence of more than a decade, but it subsequently did not take as much time to reemerge. During the first few months of 2005, a large outbreak of filoviral-like hemorrhagic fever began in the Uige Province in northern Angola. The immediate thought was that this outbreak was likely to have been caused by EBOV because of the geographic proximity to past outbreaks of EBOV infection, coupled with the apparent high case-fatality rates. However, early in 2005, this outbreak was found to be caused by a new strain of MARV (MARV Angola [MARV-Ang]), with 227 deaths recorded among 252 case patients [8]. The reasons for the increased lethality of this new strain of MARV are presently unknown but are of significant concern.

Severe coagulation disorders are one of the most prominent features of filoviral infection. For Zaire EBOV (ZEBOV), we previously showed that the procoagulant tissue factor (TF) contributes to developing coagulopathy [9] and that recombinant nematode anticoagulant protein c2 (rNAPc2), an inhibitor of the TF pathway, provided partial postexposure protection to rhesus macaques [10]. Survival and prolongation of disease course in these rNAPc2-treated animals was associated with attenuation of the coagulation and proinflammatory responses. Animals responding to treatment showed reduced plasma levels of D-dimers, less-prominent fibrin deposits in tissues, and lower plasma levels of interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1. rNAPc2 also was shown to dampen the proinflammatory response in cases of human endotoxemia [11] and in healthy human subjects in whom coagulation was deliberately provoked by a stimulant [12]. The antithrombotic potential of rNAPc2 has been demonstrated in phase II trials in the clinical settings of orthopedic surgery [13] and coronary revascularization [14]. In this study, we evaluated the pathogenicity of MARV-Ang in rhesus macaques and also assessed the efficacy of rNAPc2 against this new MARV strain.

## MATERIALS AND METHODS

**Animal studies.** Twelve healthy adult rhesus macaques (*Macaca mulatta*) weighing 3–8 kg were used in this study, which was done in 2 parts. An initial study was performed to confirm the virulence of MARV-Ang. In this first study, 3 macaques (control animals 1–3) were inoculated, by intramuscular injection, with 1000 pfu of MARV-Ang (provided by P. Rollin, Centers for Disease Control and Prevention, Atlanta, GA). The MARV-Ang strain used was originally obtained in 2005 from

serum samples from a fatally infected human [15] and was passed twice in Vero cells. After the initial study to confirm the virulence of MARV-Ang, the second study evaluated the utility of rNAPc2 as a postexposure treatment modality. The regimen followed was identical to that described elsewhere for ZEBOV infection [10]. In brief, 9 animals were exposed to 1000 pfu of MARV-Ang by means of intramuscular injection. Of these 9 animals, 6 animals (subjects 1–6) were treated with rNAPc2 by subcutaneous injection (~30 µg/kg body weight, once daily; obtained previously from Corvas International), beginning ~10 min after viral challenge and continuing to 14 days after exposure to MARV-Ang. Three macaques served as experimental control animals (4–6) and received an equivalent volume of sterile PBS in accordance with the same regimen used for the rNAPc2-treated animals. Blood samples were collected before challenge and on days 3, 6, 10, and 14 after challenge; blood samples also were collected at the time of death, when possible.

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to the principles stated in the *Guide for the Care and Use of Laboratory Animals* [16]. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (Rockville, MD).

**Virus detection.** Levels of infectious MARV-Ang were determined by plaque assay on Vero E6 cells from all blood samples and selected tissues, as described elsewhere for MARV [17, 18].

**Hematology, serum biochemistry, and coagulation tests.** Hematology and serum biochemistry were analyzed as described elsewhere, by use of a laser-based hematologic analyzer (Coulter Electronics) and a Piccolo Point-of-Care Blood Analyzer (Abaxis), respectively. Plasma levels of D-dimers and protein C were measured as detailed in other study reports [9, 10].

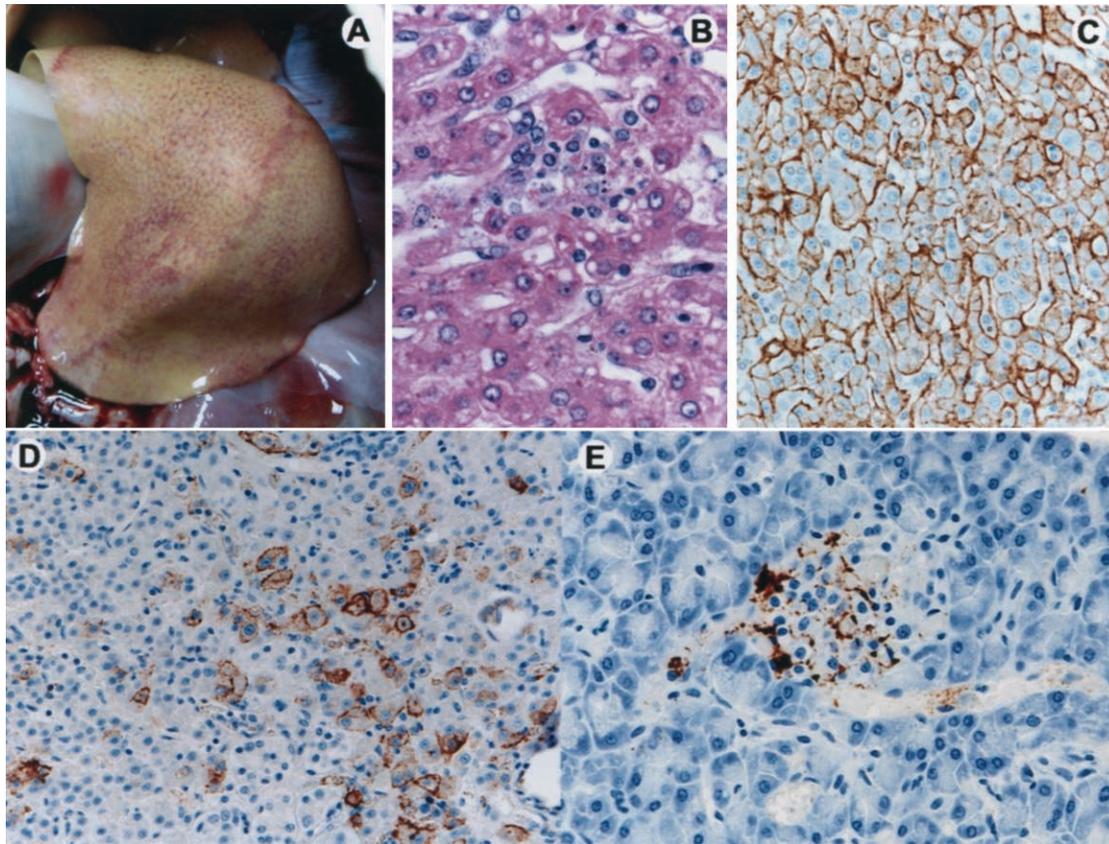
**Cytokine and chemokine production.** Cytokine and chemokine levels in monkey plasma were determined by use of a human cytokine multiplex-25 bead-array assay kit (BioSource) for the Bio-Plex 200 system (Bio-Rad), in accordance with the manufacturer's directions. The cytokines and chemokines assayed included eotaxin, granulocyte-macrophage colony-stimulating factor, interferon (IFN)-α, IFN-γ, IL-1β, IL-1 receptor antagonist, IL-2, IL-2 receptor, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40/p70, IL-13, IL-15, IL-17, IFN-γ-inducing protein-10, MCP-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, IFN-γ-induced monokine, RANTES, and tumor necrosis factor-α.

**RNAse protection assays.** The Multiprobe RNAse Protection assay (Pharmingen) was done by using peripheral blood mononuclear cells (PBMCs) collected from monkeys before and after exposure to MARV-Ang, as described elsewhere [9, 19]. Levels of mRNA expression were measured by analysis of the band intensities from a Phosphor screen on a Typhoon

**Table 1. Clinical findings for rhesus macaques infected with Marburg virus Angola.**

Animal	Day 3	Day 6	Day 7 or 8	Day 9 or 10	Day of death
<b>Subject</b>					
1	...	Lymphopenia, thrombocytopenia ALP↑, ALT↑, AST↑↑↑	Mild rash, anorexia	...	9
2	...	Fever, anorexia, lymphopenia ALP↑, ALT↑↑↑, AST↑↑↑, GGT↑, TBIL↑↑	Mild rash, anorexia	...	9
3	...	Anorexia, lymphopenia, thrombocytopenia	...	...	Survived
4	Fever	Anorexia, lymphopenia ALT↑, AST↑↑↑	Mild rash, anorexia	...	9
5	Lymphopenia	Fever, lymphopenia ALP↑, AST↑↑↑, GGT↑	Mild rash, anorexia	...	9
6	Lymphopenia	Fever, anorexia ALT↑↑↑, AST↑↑↑	Mild rash, anorexia	Mild rash, anorexia, leukocytosis ALT↑↑↑, AST↑↑↑, GGT↑, BUN↑, CRE↑	9
<b>Control</b>					
1	Lymphopenia	Fever, anorexia, lymphopenia ALP↑↑↑, ALT↑↑↑, AST↑↑↑, GGT↑↑, TBIL↑	Severe rash, anorexia, leukocytosis ALP↑↑↑, ALT↑↑↑, AST↑↑↑, GGT↑↑↑, TBIL↑↑↑, BUN↑, CRE↑, decreased GLU	...	8
2	Lymphopenia	Fever, moderate rash, anorexia ALP↑↑↑, ALT↑↑↑, AST↑↑↑, TBIL↑↑↑, CRE↑	Severe rash, anorexia, leukocytosis ALP↑↑↑, ALT↑↑↑, AST↑↑↑, TBIL↑↑↑, BUN↑↑↑, CRE↑↑↑	...	7
3	Lymphopenia	Fever, anorexia, lymphopenia ALP↑, ALT↑↑↑, AST↑↑↑, TBIL↑	Severe rash, anorexia, leukocytosis ALP↑↑↑, ALT↑↑↑, AST↑↑↑, TBIL↑↑↑, BUN↑, CRE↑↑↑	...	8
4	...	Fever, anorexia, lymphopenia ALP↑, ALT↑↑↑, GGT↑, TBIL↑	Moderate rash, anorexia, thrombocytopenia ALT↑↑↑, GGT↑, TBIL↑↑↑	...	7
5	...	Fever, mild rash, lymphopenia ALP↑, ALT↑↑↑, GGT↑↑, TBIL↑	...	...	7
6	Fever, lymphopenia	Fever, moderate rash, anorexia, lymphopenia ALP↑↑, ALT↑↑, AST↑↑↑, GGT↑↑↑, TBIL↑	...	...	7

**NOTE.** Fever was defined as a temperature >1.3°C higher than baseline or at least 0.8°C higher than baseline and ≥39.7°C. Rash was defined as follows: mild, focal areas of petechiae covering <10% of the skin; moderate, focal areas of petechiae covering 10%–40% of the skin; and severe, focal areas of petechiae and/or echymosis covering >40% of the skin. Lymphopenia and thrombocytopenia were defined as a ≥35% decrease in numbers of lymphocytes and platelets, respectively. Leukocytosis was defined as a ≥2-fold increase in white blood cells (WBCs) over baseline and also as a WBC count >11,000. †, 2–3-fold increase; ††, 4–5-fold increase; †††, >5-fold increase. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CRE, creatinine; GGT, γ-glutamyltransferase; GLU, glucose; TBIL, total bilirubin.



**Figure 1.** *A*, Gross pathology of liver of Marburg virus Angola (MARV-Ang)-infected rhesus macaque, showing severe reticulation and discoloration. *B*, Hepatocellular degeneration and necrosis with inflammation. *C*, Abundant immunopositive cells (*brown*) in liver of an untreated MARV-Ang-positive control animal at day 8 postinfection. *D*, Immunopositive cells (*brown*) in the adrenal medulla of a MARV-Ang-positive control animal at day 7 postinfection. *E*, Immunopositive cells (*brown*) in the islet of the pancreas of a MARV-Ang-positive control animal at day 7 postinfection. Original magnifications,  $\times 40$  (*B*);  $\times 20$  (*C-E*).

8600 variable-mode imager (Molecular Dynamics). Band intensities were analyzed by use of ImageQuant software (Molecular Dynamics). Changes in TF mRNA expression were calculated on the basis of the ratio to the expression of the housekeeping gene L32.

**Flow cytometry.** Approximately  $1 \times 10^6$  PBMCs were stained for cell-surface markers and TF expression. TF staining was done by incubation of cells with an anti-TF mouse monoclonal antibody against human TF (American Diagnostica), followed by a fluorescein isothiocyanate-labeled goat anti-mouse antibody (Sigma-Aldrich). Cell-surface staining was done as described elsewhere [20], to confirm gating and cell populations. Data are presented as percentage positive cells of the gated cell population. Samples were run on a FACSCalibur flow cytometer and were analyzed by use of BD Cellquest Pro software (BD Biosciences).

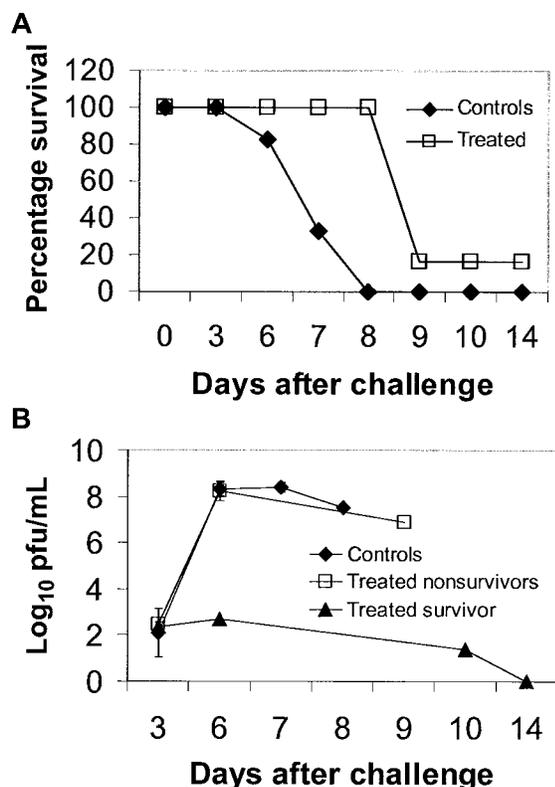
**Histopathology and immunohistochemistry.** Tissue samples were collected and fixed by immersion in 10% neutral buffered formalin, and samples were processed for histopathology, phosphotungstic acid hematoxylin (PTAH) staining to

demonstrate polymerized fibrin [9, 10], and immunohistochemistry [19], as previously described, using a mouse monoclonal antibody against MARV glycoprotein (III 5D7 [21]; 1:4000 dilution).

**Statistical analysis.** Data collected during this study were analyzed by means of the log-rank test, for comparison of survival rates, and the Wilcoxon rank sum test, for comparison of mean time to death. Mean levels of soluble mediators were compared by use of Student's *t* test.

## RESULTS

**Evaluation of MARV-Ang infection in rhesus macaques.** To investigate the pathogenic potential of MARV-Ang, 3 rhesus macaques (control animals 1–3) were experimentally infected, by intramuscular injection, with 1000 pfu of MARV-Ang. Results of previous studies that used other strains of MARV for infection of rhesus macaques and similar experimental conditions (i.e., subcutaneous or intramuscular injection of doses estimated to be between 500 and 10,000 pfu) have suggested



**Figure 2.** A, Chart showing survival over time in the postexposure treatment study of Marburg virus Angola (MARV-Ang) infection. B, Plasma viremia levels in rhesus macaques after MARV-Ang challenge, at the indicated time points.

that most rhesus macaques should begin showing clinical evidence of infection around day 4 or 5 and usually die between days 9 and 12 after challenge [6, 18, 22]. However, in this initial pilot study, disease appeared to progress more rapidly: all 3 animals began to show clinical signs of illness by day 3 (table 1). Characteristic features of MARV infection, including fever, cutaneous rashes, lymphopenia, and/or elevations in circulating levels of enzymes associated with organ dysfunction, were prominent in all animals by day 6. Thrombocytopenia, which is a prominent feature of Ebola hemorrhagic fever (EHF) in nonhuman primates [9, 10, 19, 23, 24], was not as prevalent in these animals. However, modest declines in levels of platelets (by 30%–40%), which is consistent with findings in other MARV studies of macaques [22], were seen at day 6 after infection. Decreases in plasma levels of protein C and concomitant increases in plasma levels of D-dimers were observed in the MARV-Ang-infected macaques but did not appear to be as marked as previously described for ZEBOV-infected macaques [9, 10]. Increased levels of TF transcripts were detected in PBMCs of the MARV-Ang-infected macaques but appeared to develop later in the disease course than has been reported for ZEBOV-infected macaques [9, 10]. All 3 of the MARV-

infected animals succumbed to challenge; 1 macaque died on day 7 (control animal 2) and 2 macaques died on day 8 (control animals 1 and 3).

In general, macroscopic and microscopic lesions were similar to findings previously described for MARV infection in humans and/or nonhuman primates [25–30]. However, the liver appeared to be a bit more affected by MARV-Ang (higher organ infectivity titers and more evidence of necrosis and inflammation) than has been observed in MARV Musoke-infected animals (T.W.G., unpublished data) and much more involved than the liver in ZEBOV-infected macaques [19, 23]. In brief, the livers were severely reticulated and discolored (figure 1A). Histologic findings included some hepatocellular degeneration and necrosis, with occasional neutrophilic and monocytic inflammation (figure 1B), in most of these animals. Although there is a significant paucity of immunohistochemical data from tissues of humans and nonhuman primates infected with MARV, the distribution of antigen in these macaques was consistent with findings reported for a human case of MARV infection (strain Ravn) [28]. Immunohistochemical staining for MARV antigens was particularly prominent in the liver (figure 1C) and splenic red pulp of all the animals. Similarly, organ infectivity titration showed that the liver and spleen had particularly high loads of infectious MARV-Ang, with mean titers of 9.1 and 8.8 log<sub>10</sub> pfu/g, respectively, for untreated control animals. Mean organ infectivity titers for MARV-Ang in other tissues ranged from 5.3 to 8.4 log<sub>10</sub> pfu/g (data not shown). Immunopositive staining was prominent in hepatocytes (figure 1C) and Kupffer cells in liver and also in monocytes, macrophages, and fibroblasts in most tissues examined. In addition, some animals showed other lesions consistent with findings for the human case of MARV infection, including viral infection of adrenal cortical and medullary cells (figure 1D) and pancreatic islet cells (figure 1E).

We also investigated the cytokine and chemokine responses of the MARV-Ang-infected animals, since many of these inflammatory mediators play an important role in the interaction between coagulation and systemic inflammation during infections (reviewed in [31, 32]). Increased levels of several cytokines and chemokines were detected at terminal stages of disease in all animals, including IL-6, MCP-1, IL-1 $\beta$ , and eotaxin.

**Postexposure treatment of MARV-Ang-infected rhesus macaques with rNAPc2.** To test the concept that a blockade of the TF pathway may be beneficial after MARV-Ang infection, we next treated 6 rhesus macaques with subcutaneous injections of rNAPc2 (30  $\mu$ g/kg body weight, once daily; subjects 1–6) and 3 rhesus macaques with sterile saline (control animals 4–6), beginning ~10 min after MARV-Ang challenge and continuing through day 14 after exposure. Consistent with the results from our pilot experiment, all 3 macaques treated with placebo in this study died on day 7 after MARV-Ang challenge.



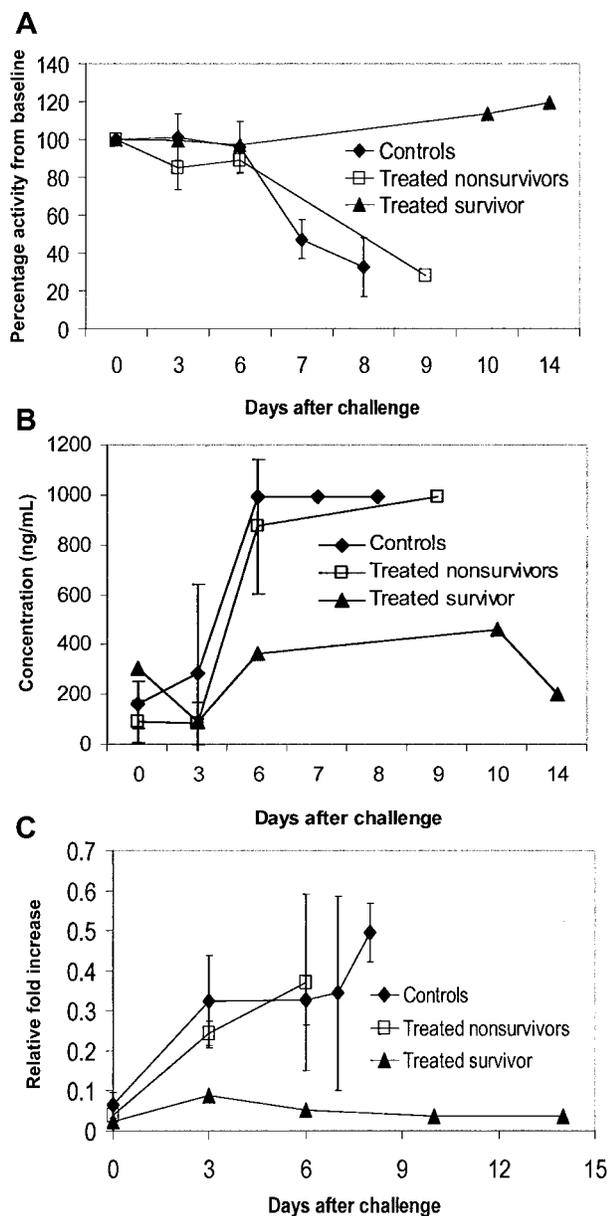
**Figure 3.** A, Petechial rash evident on the head and neck of a rhesus macaque infected with Marburg virus Angola, at day 8 postinfection. B and D, Petechial rash evident on the right arm of an untreated MARV-positive macaque (control), at days 6 and 7 postinfection, respectively. Note the increase in severity of the rash between days 6 and 7. C, Mild petechial rash evident on the right arm of a MARV-infected macaque treated with recombinant nematode anticoagulant protein c2, at day 9 postinfection.

In contrast, 1 of the rNAPc2-treated animals (subject 3) survived MARV-Ang challenge, whereas the remaining 5 rNAPc2-treated animals all died on day 9 (figure 2A). The animal that survived challenge has remained healthy for more than 1 year. The mean survival time for the 5 rNAPc2-treated animals that succumbed to MARV-Ang challenge was 9.0 days, compared with 7.3 days for the cohort of 6 control animals; this nearly 2-day prolongation in survival of the rNAPc2-treated macaques was statistically significant ( $P = .0005$ ).

By day 6 after challenge, animals in the rNAPc2-treated group showed clinical features of MARV infection that were consistent with those of the control animals (table 1). The primary difference between the groups was the slower development of macular rashes in the rNAPc2-treated animals versus the control animals (figure 3). Results for MARV-Ang plasma viremia are summarized in figure 2B. All animals became viremic, with little difference observed between the cohort of the 5 rNAPc2-treated animals that died and the control animals. The animal that survived (subject 3) had much lower plasma

viremia than did any of the other animals, with levels in subject 3 never exceeding  $3.0 \log_{10}$  pfu/mL; subject 3 became aviremic by day 14. In comparison, plasma viremia in the cohort of 5 rNAPc2-treated nonsurvivors and the control animals exceeded  $8.0 \log_{10}$  pfu/mL by day 6.

To determine the effect of rNAPc2 on the development of coagulopathy during MARV-Ang hemorrhagic fever, we monitored a range of factors involved in the regulation of coagulation and fibrinolysis. As with the results for plasma viremia, little difference in coagulation factors, such as protein C activity (figure 4A) and levels of D-dimers (figure 4B), was noted between the cohort of 5 rNAPc2-treated nonsurvivors and the control animals. As has been found during EBOV infection of macaques [9, 10], plasma levels of D-dimers increased in these 11 MARV-Ang-infected animals, while protein C activity substantially decreased. Conversely (and consistent with the results for plasma viremia), the sole rNAPc2-treated animal that survived MARV-Ang challenge (subject 3) had much lower plasma levels of D-dimers, while protein C activity did not change from



**Figure 4.** Effects of treatment with recombinant nematode anticoagulant protein c2 on coagulation responses during Marburg virus Angola infection of rhesus macaques. *A*, Plasma levels of protein C, measured by a chromatic hydrolysis assay. *B*, Plasma levels of D-dimers, measured by ELISA. *C*, Production of tissue factor (TF) mRNA in peripheral blood mononuclear cells. Changes in TF mRNA expression were calculated on the basis of the ratio to expression of housekeeping gene L32 and are presented as the fold increase relative to L32 expression. *A–C*, Data are mean  $\pm$  SD.

baseline values for this animal. Increased levels of TF mRNA transcripts were noted in the PBMCs of the cohort of 5 rNAPc2-treated nonsurvivors and in all the control animals by day 3 (figure 4C), whereas increased levels of TF transcripts were not observed in the rNAPc2-treated survivor (subject 3) at any sampling point. Evaluation of PBMCs by flow cytometry

showed increases in the percentage of TF-positive cells in the monocyte gate primarily at the later stages of infection (defined as day 6 or later) in all animals in this study. However, differences between the rNAPc2-treated animals and the placebo-treated control animals were not found.

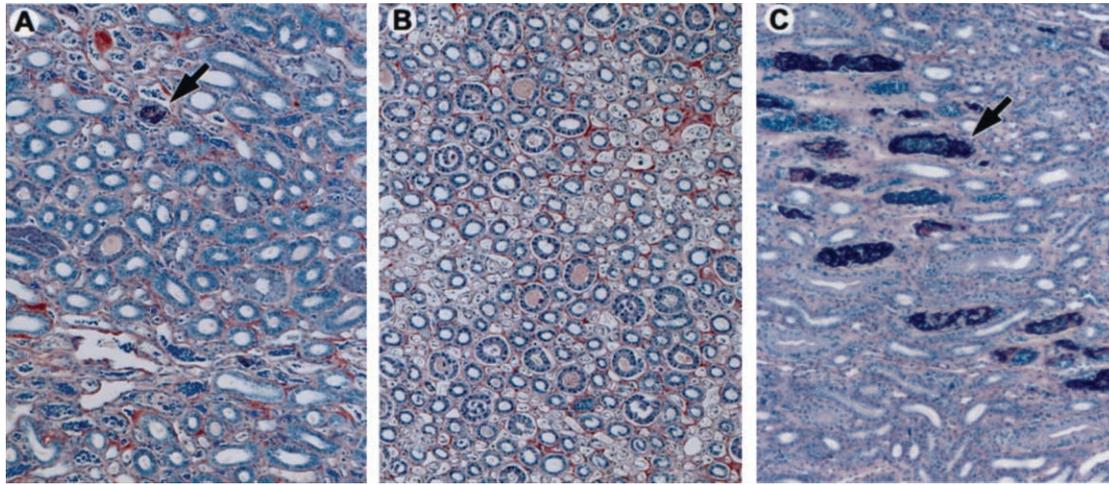
A histologic evaluation was performed on the 5 rNAPc2-treated animals that succumbed to MARV-Ang infection, to determine the extent to which this drug blocked the formation of fibrin. However, as determined by PTAH staining, no difference was seen in the amount of polymerized fibrin in the tissues of the rNAPc2-treated animals, compared with control animals. Although polymerized fibrin is a common finding in the tissues of ZEBOV-infected macaques [9, 10], polymerized fibrin was observed sporadically in the MARV-Ang-infected control or rNAPc2-treated animals in this study (figure 5).

No differences in plasma levels of cytokines and chemokines were found between the control animals and the 5 rNAPc2-treated nonsurvivors (figure 6). The only substantial difference noted was in the response of the rNAPc2-treated survivor (subject 3) versus the nonsurvivors in this study. In contrast to the nonsurvivors, subject 3 had no increase in plasma levels of IL-6, MCP-1, IL-1 $\beta$ , or eotaxin during the course of infection.

## DISCUSSION

At present, there are no licensed vaccines or treatments for MHF. The urgent need for effective countermeasures against MHF was reinforced by a recent outbreak in Angola. During the height of this outbreak, interest in evaluating the potential of rNAPc2 as a postexposure treatment for MHF was expressed by a number of international health care organizations. This interest was driven by studies of rhesus macaques that showed that the TF-pathway inhibitor rNAPc2 had some postexposure efficacy against EHF and because rNAPc2 has been safely used in a variety of medical applications [13, 14]. In the current proof-of-concept study, we evaluated the efficacy of rNAPc2 as a postexposure treatment for MARV-Ang hemorrhagic fever by administering rNAPc2 to rhesus macaques shortly after challenge with MARV-Ang. The results of this study (17% survival; 1.7-day increase in mean time to death) were not as impressive as the results obtained in studies of ZEBOV-infected rhesus macaques treated with rNAPc2 (33% survival; 3.4-day increase in mean time to death), even though the study design was identical. Nonetheless, survival and a beneficial effect were achieved in an otherwise uniformly lethal model of virus challenge.

Although there are many commonalities in the pathogenesis of EHF and MHF in macaques, we observed a few potentially important differences in the MARV-Ang-infected macaques used in this study. TF was up-regulated in the MARV-Ang-infected macaques; however, levels of TF were not as striking as those noted in ZEBOV-infected macaques [9, 10]. Differ-



**Figure 5.** A, Phosphotungstic acid hematoxylin (PTAH) stain of kidney from an untreated Marburg virus Angola (MARV-Ang)-infected rhesus macaque (control) on day 8 postinfection, showing infrequent evidence of polymerized fibrin in medullary vessels (*arrow*). B, PTAH stain of kidney from a MARV-Ang-infected macaque treated with recombinant nematode anticoagulant protein c2, on day 9 postinfection, showing no evidence of polymerized fibrin in medullary vessels. C, PTAH stain of kidney from an untreated Zaire Ebola virus-infected macaque, showing an abundance of polymerized fibrin in medullary vessels (*arrow*). Original magnification,  $\times 10$  (A–C).

ences in levels of TF may have contributed to the reduced efficacy of rNAPc2 in the MARV-Ang-infected macaques, compared with ZEBOV-infected macaques. rNAPc2 acts by inhibiting the TF/factor VIIa pathway; thus, if TF is not as important in the developing coagulopathy of MARV-Ang infection in macaques, then blocking the TF pathway may not have as much of an impact on mitigating the disease. Further studies are needed in order to systematically dissect these differences in TF production.

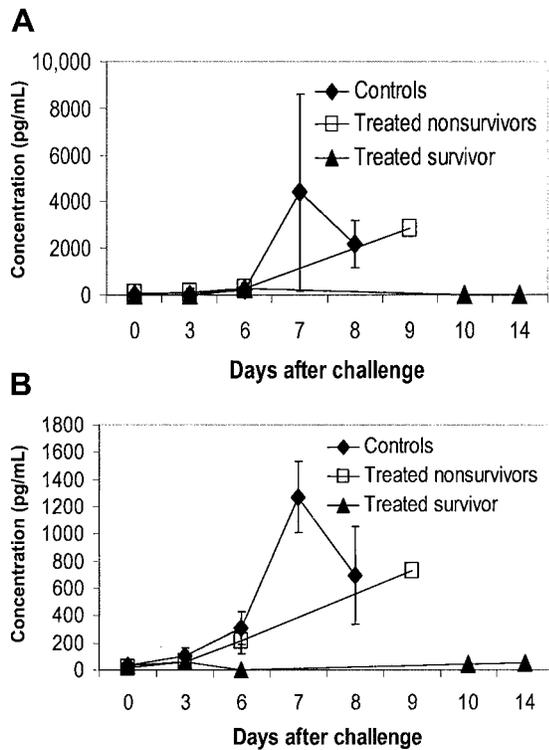
Similar to the variability in the degree of TF expression, other differences in coagulopathy were noted between the MARV-Ang-infected and ZEBOV-infected macaques. In particular, the deposition of polymerized fibrin, which is a prominent pathologic finding in tissues of ZEBOV-infected macaques [9, 10, 23], was less frequently observed in tissues of the MARV-Ang-infected macaques. Further differences include the degree of hepatocyte infection and of necrosis and inflammation of the liver, which were more prominent in the MARV-Ang-infected animals than has been reported for ZEBOV-infected macaques. In fact, mean virus titers in the livers of the MARV-Ang-infected macaques exceeded  $9.0 \log_{10}$  pfu/g, which is nearly  $2.0 \log_{10}$  pfu/g more than that typically seen in EBOV-infected macaques [19, 33]. In the MARV-Ang-infected macaques, the coagulation disorders may have been exacerbated more by the decreased synthesis of coagulation proteins, due to severe hepatocellular necrosis, than by thrombocytopenia or induction of the TF pathway of blood coagulation. rNAPc2 has no known ability to protect hepatocyte function, so the lower efficacy of rNAPc2 against MARV-Ang infection may be related to the

increased liver damage seen in the MARV-Ang-infected macaques, versus that seen in ZEBOV-infected macaques.

Another explanation for the reduced efficacy of rNAPc2 against MARV-Ang hemorrhagic fever may be the rapid disease course associated with MARV-Ang infection. The therapeutic window in our rhesus macaque model of MARV-Ang infection—that is, mean time to death of 7.3 days—was 1 day shorter than the therapeutic window for ZEBOV-infected rhesus macaques (8.3 days) [10, 34]. Whether rNAPc2 or other related modalities would have more utility for the treatment of infections caused by other MARV strains in which the disease course may be more protracted is unknown.

The reason for the apparent increased virulence of MARV-Ang in both humans and nonhuman primates is unknown. One consideration regarding our studies of macaques is the passage history of the virus strain used in this study, versus the passage history of MARV strains used in other studies. The passage history of MARV-Ang is low (i.e., 2 passages from human serum). The best direct comparison is to the Ravn strain of MARV, which also has a low passage history (2 passages from human serum), as shown when injected into 3 rhesus macaques [6]. In the study of MARV Ravn, which used a higher challenge dose of 10,000 pfu, 1 of 3 animals survived; the other animals died on days 8 and 11 after challenge. These results suggest that passage history may not explain the increased virulence of MARV-Ang relative to other MARV strains.

Growth curves comparing MARV-Ang with other MARV strains, including Musoke, Ravn, and Ci67, in primary human monocytes and macrophages showed no differences in repli-



**Figure 6.** Effects of treatment with recombinant nematode anticoagulant protein c2 on inflammatory responses during Marburg virus Angola infection of rhesus macaques. *A*, Plasma levels of interleukin-6, measured by ELISA. *B*, Plasma levels of monocyte chemoattractant protein-1, measured by ELISA. Data are mean  $\pm$  SD.

cation kinetics between these MARV strains (K.M.D.-D., unpublished data). However, plasma viremia was detected earlier in MARV-Ang-infected macaques than in macaques infected with MARV Musoke [18]. Thus, although no apparent differences in fitness were found between the MARV strains *in vitro*, we cannot eliminate the possibility that MARV-Ang was better able to replicate and/or control host immune response in non-human primates.

Although there is only a 6.8% nucleotide difference between MARV-Ang and most of the East African strains of MARV (e.g., Musoke, Ozolin, and Popp) [15], a key genetic difference somewhere in this 6.8% is among the most likely factors contributing to the apparent increased virulence of MARV-Ang in primates. Although the genetic disparity between MARV strains in the lineage containing MARV-Ang is indeed minimal, studies have shown that even single-amino acid changes can alter the cell tropism and viral pathogenicity of a number of viruses, including lymphocytic choriomeningitis virus [35, 36], dengue virus [37], severe acute respiratory syndrome virus [38], and human immunodeficiency virus [39].

In conclusion, we showed that treatment with rNAPc2 resulted in a statistically significant increase in mean time to death among rhesus macaques infected with MARV-Ang and pro-

tected 1 animal from a lethal infection. Although the results of this study were not as promising as results of a similar study using rNAPc2 to treat ZEBOV-infected rhesus macaques [10], they are nonetheless encouraging and provide a possible treatment option for MHF. Survival of 1 rNAPc2-treated animal challenged with MARV-Ang was associated with attenuated coagulation and proinflammatory responses. This finding is consistent with the survival of rNAPc2-treated, ZEBOV-infected macaques [10] and with ZEBOV-infected macaques treated with recombinant human activated protein C [40]. Moreover, in severe sepsis in humans, survival has been correlated with biomarkers that include plasma levels of protein C [41] and/or IL-6 [42]. These findings support the concept of targeting the underlying mechanisms of illness as identified through studies of pathogenesis.

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