EBOV pathogenicity has been well discussed. It is known that EBOV VP35 blocks expression of type I interferon [44,45] and that VP24 interferes with type I interferon signaling [46]. These data suggest that suppression of the innate immune response leads to dissemination of the virus throughout the body. Thus, approaches to regulate the functions of VP24 and VP35 are of interest for the development of anti-Ebola strategies.

Monocytes/macrophages infected with EBOV release tissue factor and proinflammatory cytokines/chemokines that trigger detrimental responses in EBOV-infected monkeys. The tissue factor activates a coagulation cascade and leads to intravascular fibrin formation and disseminated intravascular coagulation (DIC). DIC has frequently been found in EBOV infections in nonhuman primates [47]. Consistent with the finding that the overexpression of the tissue factor from infected monocytes/macrophages was directly involved in the pathogenesis of EBOV infection in nonhuman primates [1,2], postexposure protection by rNAPc2, an inhibitor of factor VIIa/tissue factor, was recently demonstrated in rhesus monkeys [48].

EBOV infection also impairs DC functions (e.g., secretion of protective cytokines and stimulation of lymphocytes) [10,49,50]. Increased levels of proinflammatory cytokines/chemokines from monocytes/macrophages are also prominent in EBOV infection [49]. Furthermore, bystander apoptosis of lymphocytes that are thought to be nonsusceptible to EBOV infection may result in marked lymphopenia and severe degeneration of lymphoid tissues [51,52]. Since dysfunction of these immune systems is probably critical for the pathogenesis of EBOV infection in humans and nonhuman primates [1,2], one possible approach for future drug design may be directed to restoring these immune functions.

Conclusion

Many studies have demonstrated that passive immunization with neutralizing antibodies protects mice and guinea pigs from challenge with a lethal dose of EBOV. Although in primates no sera or mAbs tested so far in the previous studies were able to provide satisfactory protection, beneficial effects such as a reduced viral load and prolonged survival, which may enable the animal to mount virus-induced immune responses, were evident. As supported by these previous studies, antibody therapy is worthy of pursuit as a treatment option for EBOV infection, and humanized mAbs to many different epitopes need to be tested.

Whether antibodies neutralize or enhance EBOV infectivity seems to depend on the balance between a number of factors, including the virus species, cell type, antibody concentration, class and epitope. The complement concentration also seems important in controlling the biological activities of the antibodies. For the practical use of passive immunization with mAbs, these characteristics should be elucidated for each antibody.

Future perspective

Available data suggest that neutralizing mAbs without binding affinity to sGP and infectivity-enhancing activity in any cell type, are required for effective antibody therapies. Importantly, if EBOV interacts with several different cellular molecules via different domains on the GP, it may be important to use a cocktail of mAbs that inhibits multiple routes of EBOV entry into cells. In addition, searching for mAbs to common epitopes among EBOV species seems to be an attractive option for the practical use of antibody therapy in the endemic area.

EBOV suppresses fundamental functions of innate immunity linked to the subsequent induction of acquired immunity such as specific antibody and cellular responses. Extensive apoptosis of lymphocytes is also an important feature of EBOV pathogenicity. Thus, strategies to improve the immune functions disrupted by EBOV infection will help to maximize the efficacy of antibody therapy in the future.

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Executive summary

Background

- Ebola virus (EBOV) causes severe hemorrhagic fever in human and nonhuman primates with high mortality rates, but effective prophylaxis and treatment for EBOV infection are currently unavailable.
- EBOV is of public health concern because of its potential for use in bioterrorism, emphasizing the need to develop effective vaccines and drugs against EBOV infection.

Functions of EBOV glycoproteins

- The EBOV glycoprotein (GP) gene encodes the structural GP and nonstructural soluble glycoprotein (sGP).
- GP is responsible for receptor binding and membrane fusion and the sole known target of neutralizing antibodies against EBOV.
- EBOV GP may interact with several different cellular molecules.
- EBOV utilizes some anti-GP antibodies to promote viral attachment to cells, which is known as antibody-dependent enhancement (ADE) of infection.
- sGP may absorb anti-GP neutralizing antibodies.

Vaccines

- Classical approaches for vaccine preparation have been generally unsuccessful to protect animals, especially nonhuman primates.
- Viral vector-based vaccines are promising candidates for use in primates.
- Induction of efficient cytotoxic T-cell and innate immune responses might be important for protection from EBOV infection.

Effects of passive immunization against EBOV infection

- Convalescent human sera have been used, concomitantly with drugs, for treatments in clinical cases, but reliable conclusions on the protective effects could not be drawn from these studies.
- In experimental conditions, treatments with polyclonal antibodies did not consistently protect animals, but reduced the severity of the disease.
- Polyclonal sera may have limited potential for prophylaxis and treatment of EBOV infection, due to the presence of ADE antibodies.
- In a mouse model, neutralizing monoclonal antibodies (mAbs) completely protected animals, whereas only partial protection was observed in guinea pig models.
- The extent of protective efficacy in rodent models was not correlated with *in vitro* neutralizing activities of the mAbs.
- Protective effects such as a reduced viral load and prolonged survival after EBOV infection were evident in some of the animal models.
- Humanized mAbs to many different epitopes need to be tested.

Future design for anti-Ebola therapies

- It may be important to use a cocktail of mAbs that inhibits multiple routes of EBOV entry into cells.
- Antibodies to common epitopes among EBOV species appear to be an attractive tool for the practical use of antibody therapy in the endemic area.
- Since dysfunction of the immune system is probably critical for the pathogenesis of EBOV infection in humans and nonhuman primates, the development of strategies to improve immune functions disrupted by EBOV infection will help to maximize the efficacy of antibody therapy.

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SUPPLEMENT ARTICLE

Epitopes Required for Antibody-Dependent Enhancement of Ebola Virus Infection

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We have shown that antibody-dependent enhancement (ADE) of infection with Zaire Ebola virus (ZEBOV) is mediated by interaction of virus-specific antibodies with Fc receptors or complement component C1q and its receptors in vitro. ADE activities of the antisera to the viral glycoprotein (GP) were virus species specific and were primarily correlated with immunoglobulin (Ig) G2a and IgM levels but not with IgG1 levels. Interestingly, compared with ZEBOV, Reston Ebola virus (REBOV) had substantially weaker potential to induce ADE antibodies. Using monoclonal antibodies, we identified ZEBOV-specific ADE epitopes. To confirm epitope specificity, we constructed a chimeric ZEBOV GP, the ADE epitopes of which were replaced with the corresponding regions of REBOV GP. We found that mouse antisera to the chimeric ZEBOV GP showed less potential to induce ADE activity than did mouse antisera to wild-type ZEBOV GP, although they retained neutralizing activity. These data suggest that GP lacking the ADE-inducing epitopes may increase the potential of GP as a vaccine antigen.

Ebola virus (EBOV) infection in primates, which is generally characterized by severe hemorrhagic manifestations, produces mortality rates higher than those associated with any of the other viral hemorrhagic fevers [1–3]. Four distinct EBOV species are known: Zaire EBOV (ZEBOV), Sudan EBOV (SEBOV), Ivory Coast EBOV, and Reston EBOV (REBOV) [1, 3]. Of these species, ZEBOV seems to be the most virulent, killing up to 90% of infected persons, whereas REBOV has been shown to be less pathogenic than ZEBOV in a nonhuman primate model [4] and has not been associated with symptomatic infection in humans [3]. Despite extensive research, the molecular basis for the extreme virulence of EBOV remains elusive.

EBOV is a filamentous, enveloped, negative-strand RNA virus. Its genome encodes at least 8 proteins, with the fourth gene from the 3′ end of the genome encoding 2 glycoproteins [1]: the envelope glycoprotein (GP), which is responsible for receptor binding and fusion of the virus with host cell membranes [5, 6], and the nonstructural secretory glycoprotein, which is released from infected cells [7, 8]. The GP functions are thought to play important roles in the pathogenesis of EBOV infection [9].

We have shown that ZEBOV exploits antiviral antibodies for their efficient entry into cells, leading to increased infectivity in vitro [10, 11]. This mechanism,

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known as antibody-dependent enhancement (ADE) of viral infection, mostly depends on the cross-linking of virus-antibody complexes through interaction with cellular Fc receptors (FcRs) [12]. FcR-mediated ADE was first described in dengue virus infection, and a possible link between ADE and the severity of dengue shock syndrome induced by secondary infection has been suggested [12]. In recent studies, we demonstrated that an additional mechanism

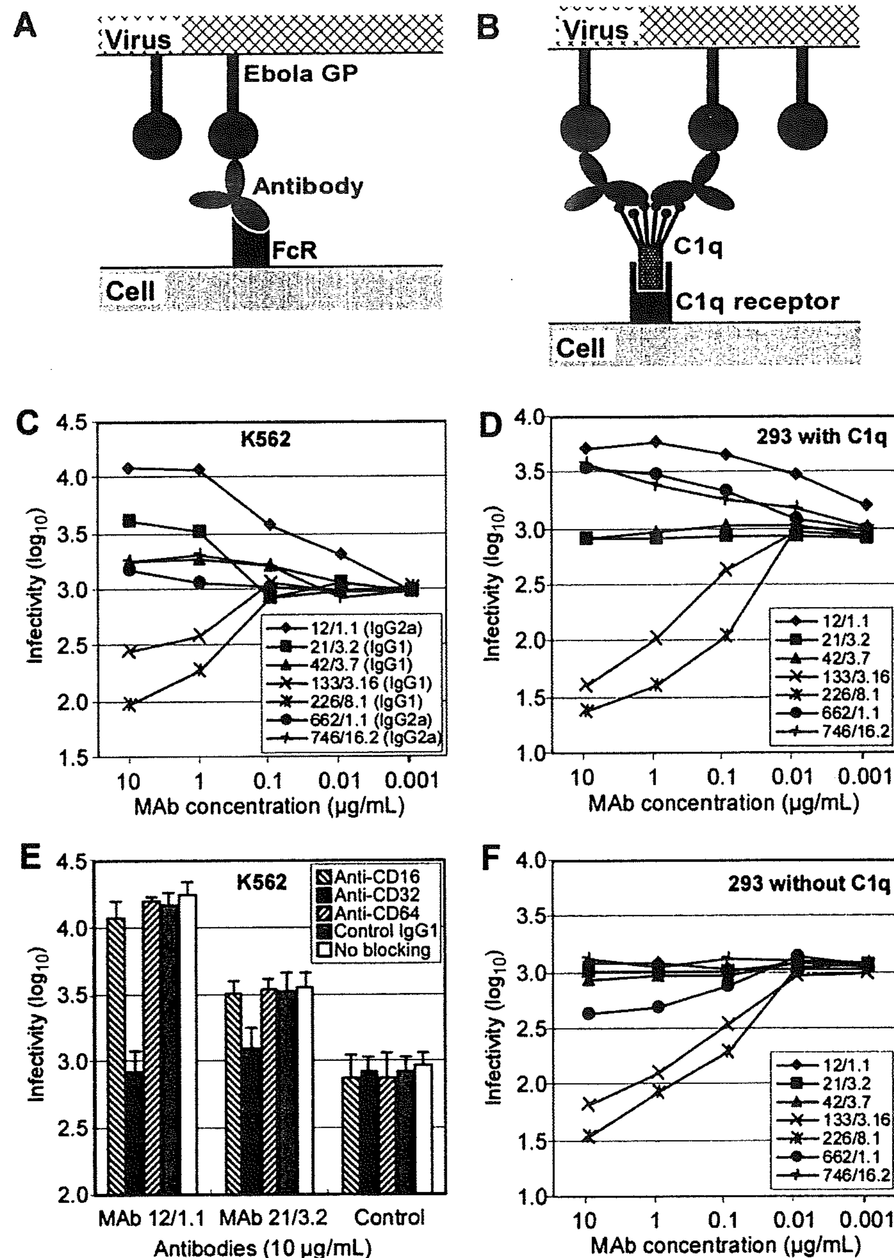

Figure 1. Antibody-dependent enhancement (ADE) of Ebola virus (EBOV) infection. Two different mechanisms for ADE of EBOV infections, in which interaction between the antibody and the Fc receptor (FcR) *(A)* or complement component C1q and the C1q receptor *(B)* promotes virus attachment to cells, are proposed. FcR-mediated ADE in K562 cells *(C)* and C1q-mediated ADE in 293 cells *(D)* were seen in the presence of monoclonal antibodies (MAbs) specific to Zaire EBOV (ZEBOV) glycoprotein (GP). Vesicular stomatitis virus pseudotyped with ZEBOV GP (10^3 infectious units/mL) was mixed with the indicated concentrations of purified antibodies and was inoculated into 10^5 K562 cells (*C* and *E*) or confluent 293 cells (*D* and *F*) in each well (96-well plate). For C1q-mediated ADE assays *(D)*, purified C1q was added to the virus-antibody mixture and then was inoculated into 293 cells. K562 cells were treated with anti-FcR MAbs or control IgG1 (10 µg/mL) during infection *(E)*.

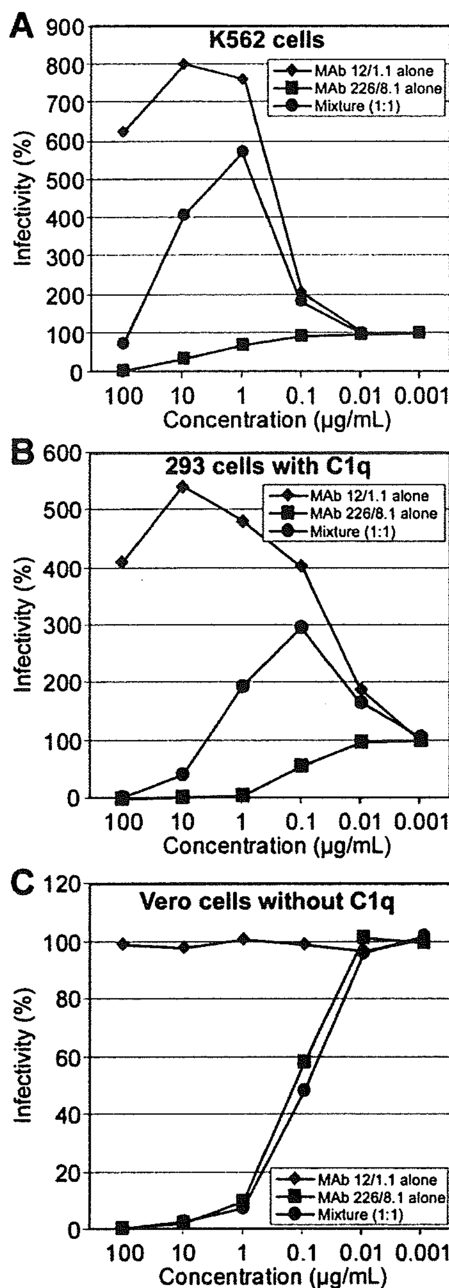

Figure 2. Antibody-dependent enhancement (ADE) versus neutralization. Vesicular stomatitis virus pseudotyped with Zaire Ebola virus glycoprotein (10^3 infectious units/mL) was mixed with the indicated concentrations of the mixture of monoclonal antibodies (MAbs) 12/1.1 and 226/8.1. Other experimental conditions were the same as those described in the figure 1 legend. *A*, Fc receptor–mediated ADE. *B*, C1q-mediated ADE. *C*, Neutralization.

underlies EBOV ADE in vitro, in which the complement protein C1q and the C1q receptor (C1qR) mediate the enhancement [11, 12]. Although FcRs are expressed exclusively on the cells of the immune system, such as the monocytes/macrophages, neutrophils, B cells, and granulocytes [13], C1qR has been identified in most mammalian cells [14, 15], suggesting a ubiquitous mechanism for ADE of EBOV infection. In the current study, we present the antibody classes and epitope specificity required for EBOV ADE, and we discuss possible roles for antibodies in the pathogenesis of EBOV.

MATERIALS AND METHODS

Viruses and cells. Vesicular stomatitis virus (VSV) pseudotyped with EBOV GP, expressing green fluorescent protein (GFP), was generated as described elsewhere [5]. Human kidney 293 and 293T cells, monkey kidney Vero E6 cells, and human chronic myelogenous leukemia K562 cells were grown in Dulbecco's modified Eagle medium and RPMI 1640, respectively, and were complemented with 10% fetal bovine syndrome, L-glutamine, and antibiotics.

Monoclonal antibodies (MAbs) and monkey plasma. Mouse MAbs were produced as described elsewhere [10]. Hybridomas producing MAbs 12/1.1 (IgG2a), 21/3.2 (IgG1), 42/3.7 (IgG1), 133/3.16 (IgG1), 226/8.1 (IgG1), 662/1.1 (IgG2a), and 746/16.2 (IgG2a) [10, 11, 16, 17] were grown in protein-free hybridoma medium II (Gibco BRL), and the antibodies were purified from the supernatants by use of protein A agarose columns (Bio-Rad Laboratories). Convalescent-phase plasma samples (obtained 3–5 months after virus challenge) were collected from monkeys that had either been vaccinated with recombinant vesicular stomatitis virus (VSV) expressing ZEBOV GP [18] or treated with recombinant nematode anticoagulant protein c2 (rNAPc2) [19] and challenged with ZEBOV or from monkeys that survived challenge with SEBOV.

Complement and anti-FcR antibodies. Human complement component C1q (Sigma) and anti-CD16, CD32, and CD64 mouse MAbs (Chemicon) were used for C1q-mediated ADE and ADE-inhibition assays performed on 293 cells and K562 cells, respectively.

Immunization of mice with virus-like particles (VLPs). Ebola VLPs were produced by transfection of pCAGGS expressing ZEBOV or REBOV GP and ZEBOV VP40 into 293T cells, as described elsewhere [20], and were purified by centrifugation through a 20% sucrose cushion. The pellet was resuspended in PBS. Protein concentrations of each VLP preparation were measured and standardized based on the optical density measured at 280 nm. Five- to six-week-old female BALB/c mice were immunized intraperitoneally or subcutaneously with 50 μg of VLPs at 3-week intervals. For hyperimmune antisera, mice were subcutaneously immunized with 100 μg of VLPs 2 times at 3-week intervals, by use of Freund complete adjuvant, and then were given an intraperitoneal booster injection (without the adjuvant) 3 weeks after the second immunization. One week after the final immunization, the mice were killed, and the serum samples were subjected to ADE and neutralization tests.

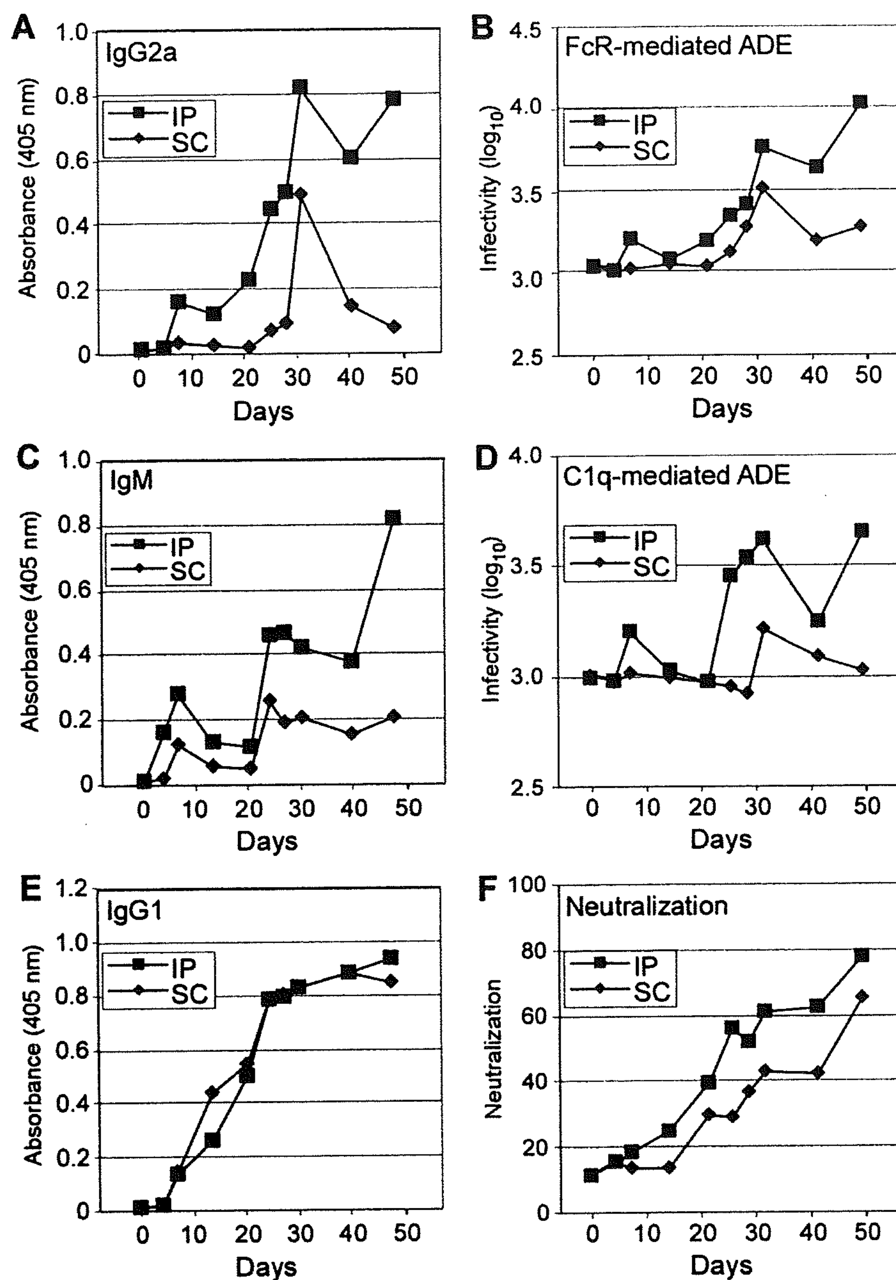

Figure 3. Kinetics of antibody levels, antibody-dependent enhancement (ADE), and neutralizing activities of the mouse serum samples. Sixty mice were immunized intraperitoneally (IP) (n = 30) or subcutaneously (SC) (n = 30) with 50 μg of Zaire Ebola virus virus-like particles (VLPs) on day 0. Mice received a booster immunization twice (on days 21 and 42). On days 0, 4, 7, 14, 21, 25, 28, 31, 41, and 49, three mice from each group were killed, and diluted serum samples (1:100) were subjected to ELISAs and ADE and neutralization tests. Results are expressed as the mean values for 3 mice. FcR, Fc receptor; Ig, immunoglobulin.

ELISA. ZEBOV GP–specific antibodies in serum samples were detected by ELISA performed as described elsewhere. Each well was coated with recombinant ZEBOV GP lacking the transmembrane domain and the cytoplasmic tail [21]. The reactions were detected by goat anti-mouse IgG1, IgG2a, and immunoglobulin (Ig) M (Bethyl Laboratories) conjugated to horseradish peroxidase.

Virus titration. The infectivity of pseudotyped VSV was determined by counting the GFP-positive cells as described elsewhere [5, 22]. In ADE assays, virus was appropriately diluted to provide 10^3 infectious units/mL and was mixed with antibodies. For detection of C1q-mediated ADE, Dulbecco's modified Eagle medium containing purified C1q (50 μg/mL) was used for dilution of the virus and antibodies. In neutralization

Table 1. Monkey immune plasma used in the present study.

Monkey	Challenge	Species	Treatment	Status
1	ZEBOV	Cynomolgus	Vaccinated with VSV-Z	Survived
2	ZEBOV	Cynomolgus	Vaccinated with VSV-Z	Survived
3	ZEBOV	Cynomolgus	Vaccinated with VSV-Z	Survived
4	ZEBOV	Cynomolgus	Vaccinated with VSV-Z	Survived
5	ZEBOV	Rhesus	rNAPc2	Survived
6	ZEBOV	Rhesus	rNAPc2	Survived
7	ZEBOV	Rhesus	None	Survived
8	SEBOV	Cynomolgus	None	Survived
9	SEBOV	Cynomolgus	None	Survived

NOTE. rNAPc2, recombinant nematode anticoagulant protein c2; SEBOV, Sudan Ebola virus; VSV-Z, vesicular stomatitis virus expressing ZEBOV glycoprotein; ZEBOV, Zaire Ebola virus.

assays, the relative percentage of infected cells was determined to be the number of infected cells in the presence of either control mouse or control human serum samples set to 100%.

RESULTS

ADE and neutralization by GP-specific MAbs. We first examined the ADE (figure 1*A* and 1*B*) and neutralizing activities of 7 ZEBOV GP–specific MAbs that recognized discrete epitopes [10, 11, 16]. The rates of infectivity of VSV pseudotyped with ZEBOV GP (VSV-ZaireGP) were determined using K562 and 293 cells in the presence of MAbs. In K562 cells, 5 of the 7 MAbs showed dose-dependent ADE activity (figure 1*C*). Of these MAbs, MAbs 12/1.1 (IgG2a) and 21/3.2 (IgG1) had ADE activity that was more significant (increases in virus infectivity of ~17- and ~4-fold, respectively) than that of the other MAbs. The enhancement by these MAbs was completely blocked by an MAb to human CD32 (FcγRII) but not by MAbs to CD16 (FcγRIII) and CD64 (FcγRI), suggesting a major contribution of FcγRII to the enhanced infectivity of the virus in K562 cells (figure 1*E*).

We previously demonstrated FcγR-independent but complement component C1q-mediated ADE of EBOV infection [10, 11] (figure 1*B*). Thus, we tested these MAbs for this unique ADE activity in 293 cells lacking detectable FcR [11]. We confirmed that MAbs 12/1.1 (IgG2a), 662/1.1 (IgG2a), and 746/16.2 (IgG2a) significantly enhanced VSV-ZaireGP infectivity in a dose-dependent manner only in the presence of purified C1q (figure 1*D* and 1*F*) [11]. None of the other antibodies showed C1q-mediated ADE activity in 293 cells. MAbs 133/3.16 and 226/8.1 consistently neutralized virus infectivity, regardless of the cell type used and the presence of C1q (figure 1*C*, 1*D*, and 1*F*).

Our previous data suggested that different types of antibodies were produced in parallel in EBOV infection and that serum antibodies represented a mixture of neutralizing, enhancing antibodies and nonneutralizing, nonenhancing antibodies [10]. Virus infectivity might be neutralized or enhanced by these serum antibodies, depending on the antibody concentration, the cell type, and the presence of complement C1q. To examine this hypothesis, we prepared a mixture of the representative MAbs for neutralization (MAb 226/8.1) and ADE (MAb 12/1.1), and we tested the activity of this mixture under different experimental conditions (figure 2). We found that neutralizing activity was detected only at high concentrations of the MAb mixture and that both types of ADE (i.e., FcγR- and C1q-mediated ADE in K562 and 293 cells, respectively) could be detected at "subneutralizing concentrations" of the antibodies (figure 2*A* and 2*B*). In the absence of FcγR and C1q (i.e., in Vero E6 cells without C1q), the mixture showed only neutralizing activity in a dose-dependent manner, such as that noted in association with MAb 226/8.1 alone, whereas MAb 12/1.1 alone showed no effect in this condition (figure 2*C*). These results suggest that, if an anti-GP polyclonal serum sample possesses a high concentration of GP-specific antibodies (e.g., 100 μg/mL in a mass), it likely has neutralizing activity irrespective of the cell type, whereas lower concentrations (e.g., 1 μg/mL) of the antibody tend to swing its apparent activity to the ADE phenotype. Importantly, these results also indicate that standard neutralization tests using Vero E6 cells always fail to detect ADE activity, because cellular FcγR or supplemented C1q and its ligands on the target cells are required to readily detect EBOV ADE in in vitro serological assays.

Antibody levels and classes correlated with ADE or neutralizing activities of serum. We then produced mouse antisera to ZEBOV GP by means of intraperitoneal and subcutaneous immunization with ZEBOV VLP, and we compared antibody responses for ADE and neutralization (figure 3). We found that ADE activity was primarily correlated with IgG2a and IgM levels in serum samples. The kinetics of IgG2a and IgM in serum were almost completely associated with FcR- (figure 3*A* and 3*B*) and C1q-mediated (figure 3*C* and 3*D*) ADE activity, respectively, throughout the experimental periods. Interestingly, intraperitoneal booster immunization on day 42 increased IgG2a and IgM levels in parallel with both types of ADE activity, whereas subcutaneous booster immunization diminished these antibody responses. By contrast, neutralizing activity was well associated with the kinetics of IgG1 in serum samples, and no significant difference was noted between intraperitoneal and subcutaneous immunization (figure 3*E* and 3*F*). These data suggest that ADE activities of anti-GP serum were caused by IgG2a levels—but not IgG1 antibody levels—and that the antibody response toward the Th1 phenotype was associated with ADE.

ADE and neutralizing activities in convalescent-phase serum samples obtained from monkeys. To determine whether

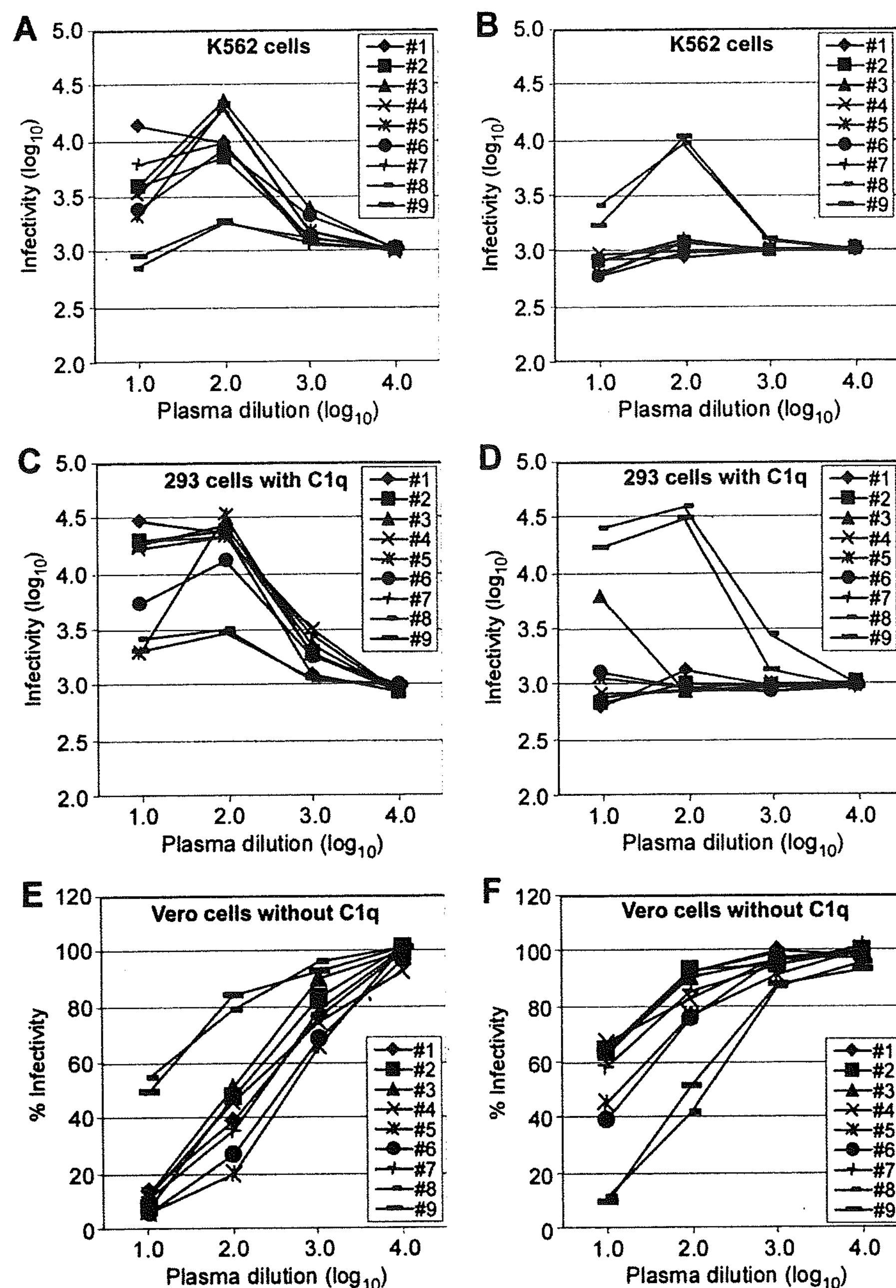

Figure 4. Antibody-dependent enhancement and neutralization by convalescent-phase serum samples obtained from monkeys. Vesicular stomatitis virus (VSV) pseudotyped with Zaire Ebola virus (EBOV) glycoprotein (GP) *(A, C,* and *E)* or VSV pseudotyped with Sudan EBOV GP *(B, D,* and *F)* was mixed with indicated dilutions of monkey serum samples (see table 1), incubated for 1 h at room temperature, and inoculated into K562 *(A* and *B)*, 293 *(C* and *D)*, or Vero E6 cells *(E* and *F)*. Other experimental conditions were the same as those described in the figure 1 legend.

EBOV infection indeed induces both ADE and neutralizing antibodies in primates, we tested convalescent-phase serum samples obtained from monkeys that survived challenge with ZEBOV or SEBOV (table 1). In K562 cells, all the serum obtained from ZEBOV-infected monkeys (monkeys 1–7) significantly enhanced VSV-ZaireGP infectivity at 1:10 and 1:100 dilutions of the serum samples, whereas enhancement by serum from SEBOV-infected monkeys (monkeys 8 and 9) was limited (figures 4*A*), indicating the presence of EBOV species–specific antibodies for FcR-mediated ADE. Accordingly, serum samples obtained from SEBOV-infected monkeys (monkeys 8 and 9) enhanced VSV-SudanGP but not VSV-ZaireGP (figure 4*B*). Similarly, species-specific C1q-mediated ADE was observed on 293 cells (figure 4*C* and 4*D*). In contrast, when Vero E6 cells were used for the assays in the absence of C1q, these serum samples showed neutralizing activity (figure 4*E* and 4*F*). Cross-

A

ADE epitopes on ZEBOV GP

MAb	Position	Amino acid sequence
12/1.1	462-501	NNNTHHQDTGEESASSGKLGLITNTIAGVAGLITGGRRTRR
21/3.2	331-350	GTNTTTEDHKIMASENSSAM
662/1.1	171-190	YRGTTFAEGVVAFLILPQAK
746/16.2	391-410	TPVYKLDISEATQVEQHHRR

B

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  1 MGVTGILQLPRDRFKRTSFFLWVIILFQRTFSIPLGVIHNSTLQVSDVDK
 51 LVCRDKLSSTNQLRSVGLNLEGNGVATDVPSATKRWGFRSGVPPKVVNYE
101 AGEWAENCYNLEIKKPDGSECLPAAPDGIRGFPRCRYVHKVSGTGPCAGD
151 FAFHKEGAFFLYDRLASTVIYRGTTFAEGVVAFLILSEPKKHFFSSHPLR
201 EPVNATEDPSSGYYSTTIRYQATGFGTNETEYLFEVDNLTYVQLESRFTP
251 QFLLQLNETIYTSGKRSNTTGKLIWKVNPEIDTTIGEWAFWETKKNLTRK
301 IRSEELSFTVVSNGAKNISGQSPARTSSDPISYHPPTNNSELVPTDSPPV
351 VQVHSQGREAAVSHLTTLATISTSPQSLTTKPGPDNSTHNTSEVDNNVPS
401 EQPNNTASITDNDSTASDTPSATTAAGPPKAENTNTSKSTDFLDPATTTS
451 PQNHSETAGANSSKLGTSPGSAAGPSQPGLTINTVSKVADSLSPTRKQKR
501 EAIVNAQPKCNPNLHYWTTQDEGAAIGLAWIPYFGPAAEGIYIEGLMHNQ
551 DGLICGLRQLANETTQALQLFLRATTELRTFSILNRKAIDFLLQRWGGTC
601 HILGPDCCIEPHDWTKNITDKIDQIIHDFVDKTLPDQGDNDNWWTGWRQW
651 IPAGIGVTGVVIAVIALFCICKFVF
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Figure 5. Antibody-dependent enhancement (ADE) epitopes on Zaire Ebola virus (ZEBOV) glycoprotein (GP) *(A)* and amino acid sequences of the chimeric ZEBOV GP (ZdelEP GP), the ADE epitopes of which are replaced with the corresponding region of Reston Ebola virus (REBOV) GP *(B)*. Amino acid sequences derived from REBOV GP are underlined. MAb, monoclonal antibody.

neutralization between ZEBOV and SEBOV was not considerable. These results also reinforce the importance of cell lines and experimental conditions for the detection of ADE antibodies in serum samples. Taken together, our results indicate that EBOV infection induces both ADE and neutralizing antibodies and that ZEBOV and SEBOV GPs share few common epitopes for such activity.

Identification of epitopes and generation of ZEBOV GP lacking ADE epitopes. We have shown that there is little cross-reactivity by ADE antibodies among EBOV species [10, 11]. Thus, attempts were made to identify ZEBOV-specific epitopes recognized by MAbs that enhanced virus infectivity. By use of synthetic peptides derived from ZEBOV GP amino acid sequences and chimeric GP molecules between ZEBOV and REBOV GPs [11], 4 different epitopes on ZEBOV GP were identified (figure 5*A*). Because REBOV GP has substantially weaker potential to induce ADE antibodies [10], and because the MAbs used in this study did not bind to REBOV GP (data not shown), we then undertook construction of ZEBOV GP, the ADE epitopes of which are disrupted by replacing amino acid sequences with those from REBOV GP (figure 5*B*). This chimeric (ZdelEP) GP was functionally competent, because VSV pseudotyped with ZdelEP GP (VSV- ZdelEPGP) was fully infectious in K562, 293, and Vero E6 cells.

Immunogenicity of ZdelEP GP. To evaluate the immunogenicity of ZdelEP GP, we first tested the reactivity of ZEBOV-infected monkey serum (obtained from monkeys 1–7) (table 1) to VSV-ZaireGP and VSV-ZdelEPGP, by comparing ADE and neutralization by these serum samples (figure 6*A*, 6*C*, and 6*E*). Much less ADE was observed in VSV-ZdelEPGP infection, as compared with VSV-ZaireGP (figure 6*A* and 6*C*), whereas both viruses were similarly neutralized by these serum samples (figure 6*E*), indicating that ADE antibodies produced by ZEBOV infection also targeted these epitopes. We next produced mouse antisera to ZEBOV and ZdelEP GPs, and we compared their potentials to induce ADE and neutralizing antibodies (figure 6*B*, 6*D*, and 6*F*). As expected, by both mechanisms of ADE, anti-ZEBOV GP serum samples significantly enhanced the infectivity of VSV-ZaireGP at subneutralizing concentrations of the antibody (i.e., at dilutions of 1:100–1000), whereas it showed neutralizing activity at dilutions of 1:10 (figure 6*B* and 6*D*). In contrast, anti-ZdelEP GP serum samples showed only marginal activity for both types of ADE. However, both antisera similarly neutralized VSV-ZaireGP infectivity (figure 6*F*). These results indicate that the chimeric GP had less potential to induce ADE than did wild-type ZEBOV GP, while it also maintained neutralizing epitopes.

DISCUSSION

Whether antibodies neutralize or enhance EBOV infection in cell culture seems to depend on the balance between a number of factors, including the virus species, the cell type, and the antibody concentration, class, and epitopes. The complement concentration also seems to be important in controlling the biological activities of the antibodies. If whole serum samples are used for ADE and neutralization assays, the results represent a mixture of many different effects produced by these factors. In the present study, we partly reconstructed possible factors (i.e., antibody, FcR, and C1q) in experimentally controlled conditions (figure 1 and 2), and we showed that ADE of EBOV infection could be detected, depending on the antibody concentration and presence of FcR and C1q. Accordingly, we found that 3 different activities could be detected in serum samples obtained from infected monkeys and GP-immunized mice: ADE in K562 cells, ADE in 293 cells in the presence of C1q, and neutralization in Vero E6 cells. It is noted that it seems to be difficult to observe C1q-mediated ADE activity in ordinary neutralization assays using primate kidney cells, because the diluted serum samples do not contain sufficient amounts of C1q. Similarly, FcR-mediated ADE can be seen only in the cells bearing FcR, such as K562 cells. These observations suggest that serum antibody activities during EBOV infection might be complex and that ADE activity may be considered to be one of the factors affecting the protective efficacy of vaccination or treatment for EBOV infection.

We showed that intraperitoneal immunization of mice elicited ADE antibodies more efficiently than did subcutaneous immunization, which was associated with production of IgG2a and IgM antibodies, but not IgG1 antibodies, in the serum. Serum levels of IgG2b and IgG3 were also correlated with ADE

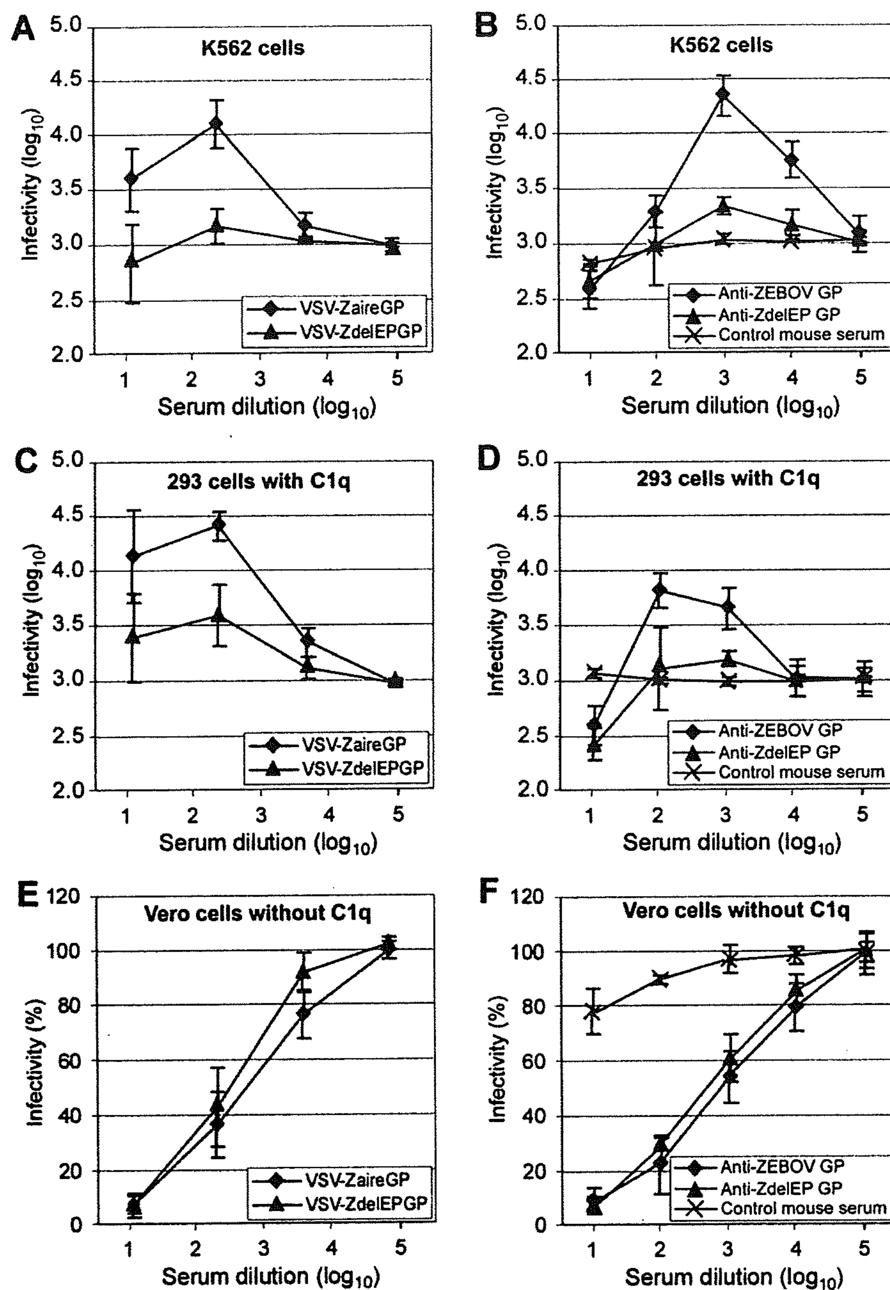

Figure 6. Antibody-dependent enhancement (ADE) and neutralization by hyperimmune serum samples obtained from mice and convalescent-phase serum samples obtained from monkeys. Tenfold diluted convalescent-phase serum samples obtained from monkeys (see table 1) were mixed with vesicular stomatitis virus (VSV) pseudotyped with Zaire Ebola virus (ZEBOV) glycoprotein (GP) (VSV-ZaireGP) or VSV pseudotyped with the glycoprotein of the chimeric ZdelEP (ZdelEPGP) *(A, C,* and *E)*, incubated for 1 h at room temperature, and inoculated into K562 *(A)*, 293 *(C)*, or Vero E6 cells *(E)*. Tenfold diluted mouse antisera to ZEBOV or ZdelEPGP were mixed with VSV-ZaireGP *(B, D,* and *F)*, incubated for 1 h at room temperature, and inoculated into K562 *(B)*, 293 *(D)*, or Vero E6 cells *(F)*. Other experimental conditions were the same as those described in the figure 1 legend. Results are expressed as means (± SDs) of data for ZEBOV-infected monkeys (monkeys 1–7) *(A, C,* and *E)* and 3 immunized mice *(B, D,* and *F)*.

activity (data not shown). It is highly likely that IgG3 and IgM play major roles in C1q-mediated ADE activity, because these classes of antibodies have a higher affinity for C1q molecules than does IgG1 [23]. Interestingly, Warfield et al. [24] showed that intraperitoneal immunization of mice with VLPs (1 μg) induced higher titers of GP-specific antibodies in ELISA than did intramuscular immunization (10 μg); however, the former did not protect mice at all. These data suggest that antibodies induced by intraperitoneal immunization may not be fully protective. Our findings may partly explain this observation and may support the notion that ADE antibodies contribute to exacerbation of the disease.

C1qR has been identified not only in the primary target cells of filoviruses (i.e., monocytes/macrophages) but also in most mammalian cells, including endothelial cells, hepatocytes, and epithelial cells [14, 15]. Importantly, because IgG and IgM antibodies are produced in patients infected with EBOV—even in nonsurvivors—during the early phase of EBOV infection [25, 26], we hypothesize that, in the middle to late phases of EBOV infection, ADE by some anti-GP IgM may facilitate rapid spread of the virus in the host by accelerating infection of the secondary target cells, such as endothelial cells and hepatocytes. However, it remains unclear whether ADE is a potential risk of disease exacerbation associated with secondary infection by EBOV.

In our previous study, we identified 2 neutralizing epitopes on ZEBOV GP [16]. The present study demonstrated 4 distinct epitopes to which ADE antibodies bound. These neutralizing and ADE epitopes were mostly located on the GP_1 subunit and were little shared among ZEBOV, SEBOV, and REBOV GPs, whereas monoclonal antibodies that had neither neutralizing nor ADE activity tended to show cross-reactivity to multiple EBOV species (data not shown). The characteristics of this array of antibodies may depend essentially on their epitopes, as well as the antibody classes, as discussed above. REBOV GP seems to have fewer epitopes involved in ADE. In the proposed mechanisms of ADE, interaction of antibodies with FcR or C1q is a requirement for ADE (figures 1*A* and 1*B*). When the conformation of the antibody-GP complex allows the Fc portion of the antibody to interact with FcR or C1q, the antibodies make a bridge between the virus and cells, facilitating access to a specific receptor for the virus. Thus, the different extent of antibody interaction with FcR or C1q between ZEBOV and REBOV GP antibodies likely depends on the epitope structure and/or conformation of the antibody-GP complex. It is also possible that glycosylation patterns affect the accessibility of the antibodies to potential ADE epitopes.

Recent progress in generating recombinant viruses from cloned cDNA has opened opportunities to design recombinant viruses that express ZEBOV GP and induce protective immune responses in a primate model [18, 27]. Available data suggest that the protective effects of such GP-based viral vector vaccines appear to depend on cytotoxic T cell responses rather than antibody responses. Although our approach, in which B cell epitopes involved in ADE are modified, may provide an option for the use of GP as a vaccine antigen, it is important to retain cytotoxic T lymphocyte epitopes for the maximal protective effect of the vaccines. Detailed epitope analyses of EBOV GP molecules for human major histocompatibility complex may be required for the design of Ebola vaccines in the future.

We have shown 2 distinct mechanisms underlying ADE of EBOV infection in vitro. Although, to our knowledge, compelling evidence for the in vivo relevance of ADE has not been provided thus far, the repeated demonstration of this phenomenon in vitro, particularly in infection with such hemorrhagic fever viruses as dengue, hantaan, and Lassa viruses [12, 28–30], continues to raise practical issues about the development of vaccines and passive prophylaxis with antiviral antibodies.

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Inhibition of Lassa and Marburg Virus Production by Tetherin[▽]

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Recently, tetherin has been identified as an effective cellular factor that prevents the release of human immunodeficiency virus type 1. Here, we show that the production of virus-like particles induced by viral matrix proteins of Lassa virus or Marburg virus was markedly inhibited by tetherin and that N-linked glycosylation of tetherin was dispensable for this antiviral activity. Our data also suggest that viral matrix proteins or one or more components that originate from host cells are targets of tetherin but that viral surface glycoproteins are not. These results suggest that tetherin inhibits the release of a wide variety of enveloped viruses from host cells by a common mechanism.

There are a number of innate host defense systems against virus infection, including interferon (IFN) and toll-like receptor signaling pathways. Cellular factors that inhibit viral replication through interactions with viral components at various steps have also been identified.

Recently, tetherin (also known as BST2, CD317, or HM1.24) was identified as a cellular factor that inhibits the release of human immunodeficiency virus type 1 (HIV-1) from infected cells (6). Tetherin is a membrane-associated protein with an N-terminal transmembrane domain, a central extracellular domain with two potential N-linked glycosylation sites, and a C-terminal glycosylphosphatidylinositol (GPI) anchor (Fig. 1A) (3, 4), which appears to prevent HIV-1 release by retaining fully formed progeny virions on the surfaces of infected cells (6, 11). Tetherin is constitutively present on the surfaces of HeLa and CEM cells, while its cell surface expression is induced by alpha IFN (IFN-α) in HEK293, 293T, HOS, HT1080, and COS-7 cells. Tetherin expression has also been reported to be stimulated by IFN in various tissues, including those of the liver, lung, placenta, heart, pancreas, kidney, skeletal muscle, and brain (1, 3), suggesting that it may function as part of IFN-induced innate immunity against enveloped viruses in vivo.

The antiviral activity of tetherin is antagonized by HIV-1 Vpu due to the downregulation of cell surface expression of tetherin by Vpu (6, 11). Previously, the IFN-α-induced cell surface retention of virus-like particles (VLPs) induced by Ebola virus matrix protein VP40 was shown to be overcome by Vpu expression (5). Thus, the release of enveloped viruses other than HIV-1 may also be inhibited by tetherin.

Lassa and Marburg viruses are emerging viruses belonging to the families *Arenaviridae* and *Filoviridae*, respectively, that cause hemorrhagic fever with high mortality rates. No approved vaccines or antiviral drugs are available to prevent or treat these viral diseases. Similar to HIV-1, both are enveloped viruses that exit the host cells by membrane extrusion, known as budding, from the plasma membrane. Therefore, having an antiviral effect against Lassa and Marburg viruses would make tetherin a potent tool for novel antiviral strategies against a wide variety of enveloped viruses.

We examined the antiviral activities of tetherin against Lassa and Marburg viruses and analyzed the characteristics required for its antiviral activity in order to gain insight into its antiviral mechanism of action.

Inhibition of Lassa and Marburg virus release by tetherin. Previously, we showed that Lassa virus Z proteins are released as VLPs when expressed alone in cells (9). To examine whether tetherin has inhibitory effects on Lassa virus release, we constructed a tetherin expression plasmid for the wild type (WT). A cDNA-encoding tetherin was amplified by PCR from the Human Lung Marathon-Ready cDNA library (BD Biosciences, San Jose, CA) and inserted into pCDNFL, which was constructed from pcDNA3.1 (Invitrogen, Carlsbad, CA) to express a protein containing a FLAG tag at the N terminus. As described previously (9), we transfected expression plasmids for Lassa virus Z and GP-C proteins to mimic Lassa virus release, along with a tetherin expression plasmid, into COS-7 and 293T cells, in which endogenous expression of tetherin is undetectable (6, 11). At 48 h after transfection, the amount of Z/GP-C-induced VLP release from the cells was analyzed by Western blotting as described previously (9, 13, 14). The exogenous expression of tetherin markedly reduced VLP release in both COS-7 and 293T cells (Fig. 1B to D). As tetherin is a cell surface protein, it may inhibit Lassa VLP release by interaction with the viral surface envelope glycoprotein GP-C on the cell surface. Therefore, we examined whether tetherin inhibits the release of VLPs induced by Z only, and a marked inhibitory effect was observed (Fig. 1E). Thus, the target of tetherin seems to be viral matrix proteins or component(s) originating from host cells, but not viral surface glycoproteins such as GP-C.

To confirm that inhibition of Lassa VLP production by tetherin occurs at the step of particle release, as observed for HIV-1, we carried out an electron microscopic analysis. As

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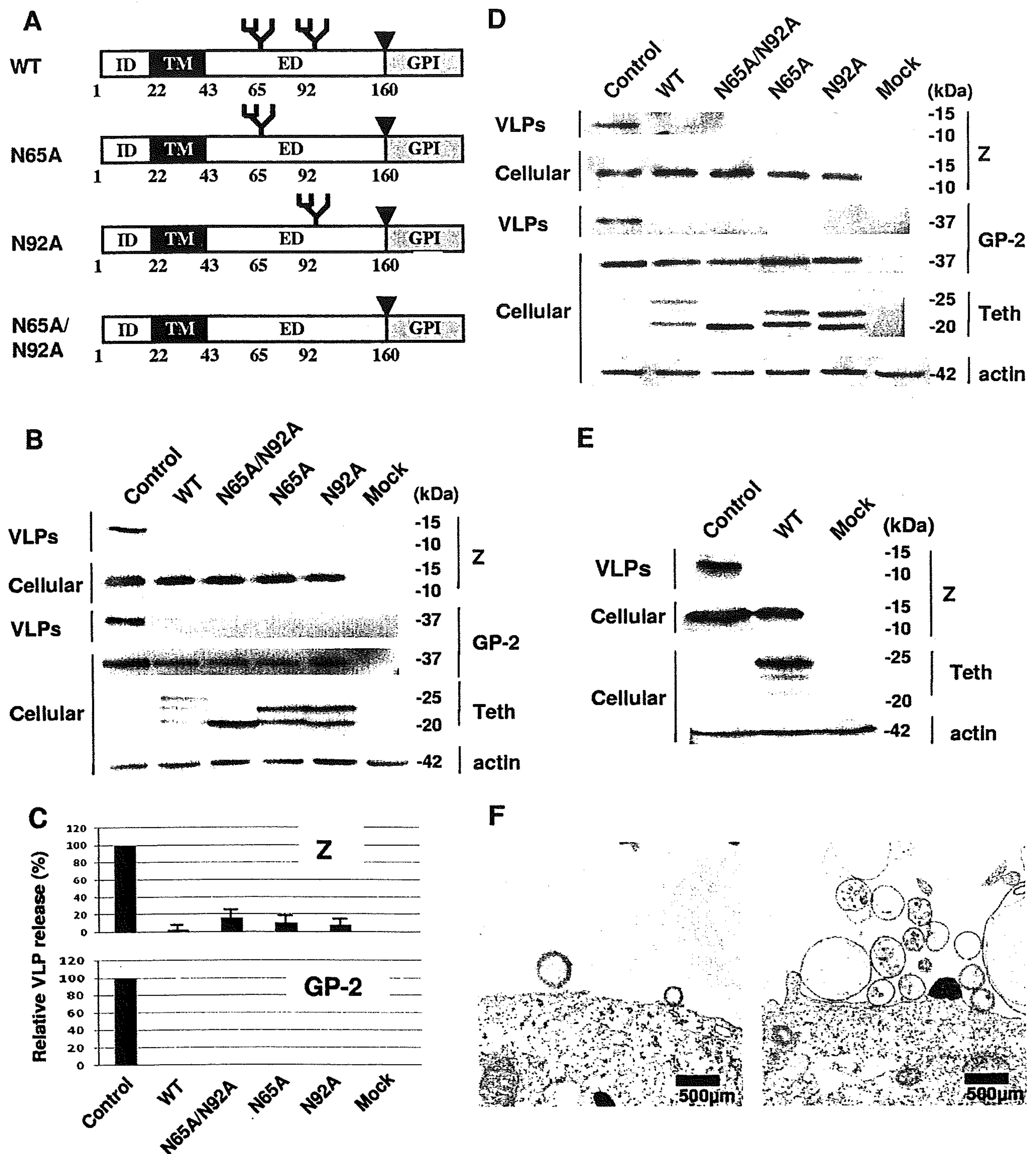

FIG. 1. Inhibitory effects of tetherin and its mutants against Lassa VLP release. (A) Tetherin (WT) contains an N-terminal intracellular domain (ID), a transmembrane domain (TM), a central extracellular domain (ED), and a C-terminal GPI anchor (GPI). Arrowheads indicate the predicted sites of cleavage prior to the addition of the GPI anchor. Tetherin possesses two potential N-linked glycosylation sites at positions 65 and 92 in the ED. N65A and N92A are mutants with the loss of a glycosylation site by an Asn-to-Ala substitution at positions 65 and 92, respectively. N65A/N92A is a nonglycosylated mutant with the loss of both glycosylation sites. (B and D) The Lassa virus Z and GP-C expression plasmids were cotransfected with the expression plasmid for WT or mutant tetherin or an empty vector (Control) into COS-7 cells (B) or 293T cells (D). Extracellular VLPs induced by Lassa virus Z/GP-C were pelleted from the culture fluids. Cell- or VLP-associated Z and GP-C (GP-2) were detected by Western blotting using rabbit anti-Z antiserum and mouse anti-GP-2 monoclonal antibody. WB using anti-FLAG antibody was also performed to examine the expression of WT and mutant tetherin in cells. WB for actin was done as the internal control. (C) The intensities of the bands for VLP-associated Z or GP-2 in panel B were quantified using a LAS3000 imaging system (Fujifilm). The level of Z or GP-2 in VLPs released from cells cotransfected with control vector was set to 100%. The data are shown as averages and standard deviations for three independent experiments. (E) COS-7 cells were cotransfected with the Lassa virus Z expression plasmid and the expression plasmid for tetherin (WT) or the empty vector (Control). VLPs induced by Z alone were examined by WB as described above. (F) 293T cells were cotransfected with pCLV-Z and the empty vector (left) or the expression plasmid for tetherin (right). At 48 h posttransfection, cells were observed by electron microscopy, which was performed as described previously (9). Mock, mock infected; Teth, tetherin. Bars, 500 nm.

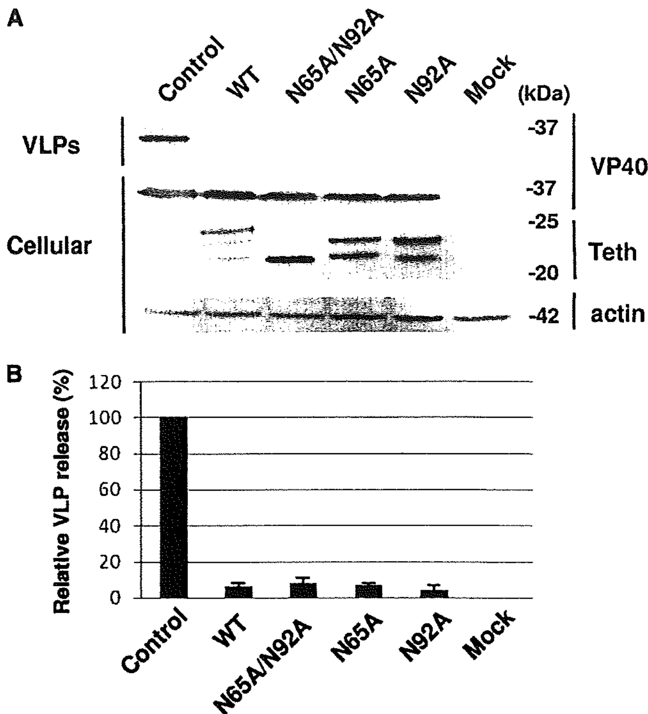

FIG. 2. Effects of WT and mutant tetherin expression on Marburg VLP production. (A) The Marburg virus VP40 expression plasmid was cotransfected with the expression plasmid for WT or mutant tetherin or an empty vector (Control) into COS-7 cells. Marburg virus VP40-induced VLP release from cells was analyzed by WB using rabbit anti-VP40 antiserum. WB using an anti-FLAG antibody was also performed to examine the expression of WT and mutant tetherin in cells. WB for actin was done as an internal control. (B) The intensities of the bands for VLP-associated VP40 in panel A were quantified as described in the legend to Fig. 1C. The level of VP40 in VLPs released from cells cotransfected with the control vector was set to 100%. The data are shown as averages and standard deviations for three independent experiments. Mock, mock infected; Teth, tetherin.

shown in Fig. 1F, retention of Lassa VLPs on the cell surfaces was observed for 293T cells expressing tetherin but not for those not expressing tetherin, indicating that tetherin inhibits the release of Lassa VLPs from cells.

To determine whether this antiviral activity of tetherin is applicable to a wide variety of enveloped viruses, we examined the inhibitory effect of tetherin on the release of Marburg virus. Marburg virus matrix protein VP40 can also release VLPs from cells solely expressing it (10). We cotransfected expression plasmids for VP40 and tetherin by using COS-7 cells and analyzed the release of VP40-induced VLPs as described previously (10). Coexpression of tetherin with Marburg virus VP40 abolished the release of VP40-induced VLPs as well as that of Lassa virus Z-induced VLPs (Fig. 2A and B), suggesting that tetherin has an inhibitory effect on release of a wide variety of envelope viruses, including HIV-1 and Lassa and Marburg viruses.

N-linked glycosylation of tetherin. The extracellular domain of tetherin has two putative N-linked glycosylation sites, N65 and N92 (Fig. 1A), which are conserved among humans, rhesus monkeys, rats, and mice, and orthologues have been identified that are actually glycosylated heterogeneously (4, 7). We examined the antiviral role of N-linked glycosylation of tetherin, as N-linked glycosylation is involved in the activities of many functional proteins. We introduced single or multiple mutations into the N-linked glycosylation sites of tetherin (Fig. 1A) and analyzed the effects of exogenous expression of these mutants on the release of Lassa virus Z/GP-C-induced VLPs. To construct expression plasmids for the N65A, N92A, and N65A/N92A mutants, Asn-to-Ala substitutions at position 65, position 92, and both of these positions, respectively, were introduced into the expression plasmid for WT tetherin by using a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). WT tetherin was detected as triplet bands, while N65A and N92A mutants and the N65A/N92A mutant were detected as double and single bands, respectively (Fig. 1B and D), indicating that the upper, middle, and lower bands of triplet forms correspond to multiple, single, and nonglycosylated forms, respectively.

In COS-7 and 293T cells as well as in the WT, the exogenous expression of any tetherin glycosylation mutant markedly reduced VLP release (Fig. 1B to D). This effect was also observed for the release of Marburg virus VP40-induced VLPs (Fig. 2). Thus, N-linked glycosylation is dispensable for the inhibition of virus release by tetherin.

Antiviral function of tetherin is antagonized by HIV-1 Vpu. Tetherin is constitutively expressed on the surfaces of HeLa cells and is downregulated and antagonized by HIV-1 Vpu (11). To determine whether the antiviral activity of tetherin against Lassa and Marburg viruses is also antagonized by Vpu, we constructed a Vpu expression plasmid. The coding region

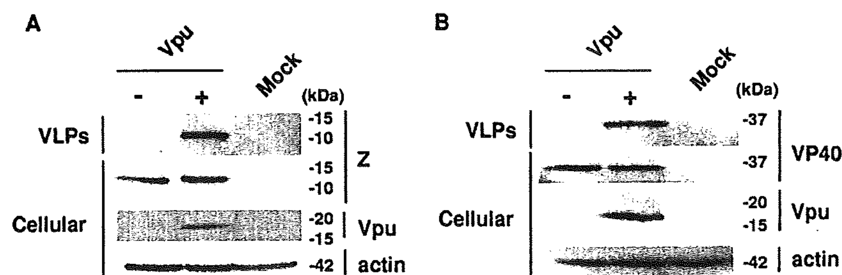

FIG. 3. Enhancement of Lassa and Marburg VLP release from HeLa cells by Vpu. The expression plasmid for HIV-1 Vpu (+) or the empty vector (−) was cotransfected with the expression plasmid for Lassa virus Z (A) or Marburg virus VP40 (B) into HeLa cells. Lassa or Marburg VLP release from HeLa cells in the absence or presence of Vpu was analyzed by WB. WB using anti-Myc antibody was also performed to examine the expression of Vpu in cells. Mock, mock infected.

of Vpu was amplified from pNL4.3 by PCR and subcloned into pBj-Myc-BUL1, which was constructed for the expression of BUL1 containing a Myc tag at the N terminus (13) by replacing BUL1 with Vpu. The Vpu expression plasmid was cotransfected into HeLa cells along with the expression plasmid for Lassa virus Z or Marburg virus VP40, and VLPs released from cells were analyzed. In the absence of Vpu expression, neither Lassa virus Z- nor Marburg virus VP40-induced VLPs were detected in the culture supernatant, while both Lassa and Marburg VLPs were released from cells coexpressing Vpu (Fig. 3A and B). Thus, as expected, Vpu significantly enhanced the production of Lassa virus Z- and Marburg virus VP40-induced VLPs from HeLa cells constitutively expressing endogenous tetherin, suggesting that tetherin endogenously expressed in HeLa cells inhibits the release of both Lassa and Marburg VLPs and that this inhibition is abolished by the expression of Vpu.

We show that tetherin inhibits Lassa and Marburg VLP release independent of N-linked glycosylation. Tetherin is induced by IFN-α in some T-cell and fibroblast cell lines and is localized to lipid rafts in the plasma membrane (8), from which most envelope virus progeny are released. Tetherin is constitutively expressed in B cells, dendritic cells, and activated T cells and plays a role in regulating B-cell growth and development (1, 2, 12). However, the precise in vivo expression pattern and physiological functions of tetherin are not known. The present study suggests that tetherin functions as a host innate antiviral system against a wide variety of viruses. Analyses of the expression pattern of tetherin in vivo may aid in understanding the specificity (tropism) of virus replication in tissues or cells. Furthermore, tetherin may have great potential for the development of antiviral strategies against a wide variety of viruses, including highly virulent emerging viruses.

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Rapid and simple detection of Ebola virus by reverse transcription-loop-mediated isothermal amplification

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Abstract

Ebola virus (EBOV) causes severe hemorrhagic fever in humans and nonhuman primates with high mortality rates. Rapid identification of the virus is required to prevent spread of the infection. In this study, we developed and evaluated a one-step simple reverse transcription-loop mediated isothermal amplification (RT-LAMP) assay for the rapid detection of *Zaire ebolavirus* (ZEBOV), the most virulent species of EBOV, targeting the trailer region of the viral genome. The assay could detect 20 copies of the artificial ZEBOV RNA in 26 min with a real time-monitoring detection, and also detect 10^{-3} FFU of the cell-culture propagated viruses. The reaction time needed to detect 10^4 FFU of ZEBOV was only 20 min. In addition, the assay was highly specific for ZEBOV. The RT-LAMP assay developed in this study is rapid, simple, highly specific, and sensitive for the detection of ZEBOV, and so may be an effective diagnostic tool. Furthermore, as this technique does not require sophisticated instrumentation, it seems very suitable for diagnosis in the field or laboratories in Ebola outbreak areas such as Central Africa.
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Keywords: Loop-mediated isothermal amplification; Ebola virus; Detection; Diagnosis

1. Introduction

The family *Filoviridae* within the order *Mononegavirales* contains two genera, *Marburgvirus* (MARV) and *Ebolavirus* (EBOV). The genus *Ebolavirus* has four species: *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Ivory Coast ebolavirus* (ICEBOV), and *Reston ebolavirus* (REBOV) (Feldmann et al., 2004). EBOV causes a lethal hemorrhagic fever in both human and nonhuman primates. Three species of EBOV, ZEBOV, SEBOV, and ICEBOV, are known to be pathogenic to humans. Among these viruses, ZEBOV seems to be the most virulent with a fatality rate of up to 90% (Mahanty and Bray, 2004).

EBOV is a non-segmented, negative-sense RNA virus. The virus genome is almost 19 kb in length and encodes seven viral structural proteins in the order nucleoprotein (NP), phosphoprotein (VP35), matrix protein (VP40), glycoprotein (GP), replication-transcription protein (VP30), matrix protein (VP24), and polymerase (L), with an additional soluble glycoprotein produced from an edited GP mRNA (Feldmann et al., 2001; Sanchez et al., 1996). The genes are flanked by 3′- and 5′-noncoding sequence, termed the leader and trailer regions, respectively.

In terms of biohazard, EBOV are classified as biosafety level (BSL) 4 agents based on their high virulence, person-to-person transmission, and the absence of effective vaccines or chemotherapeutic agents. Rapid laboratory diagnosis of Ebola

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hemorrhagic fever (EHF) is important for preventing the spread of infection (Grolla et al., 2005). Virus isolation, transmission electron microscopy, immunohistochemistry (Lloyd et al., 1999; Zaki et al., 1999), antigen capture enzyme-linked immunosorbent assay (ELISA) (Ksiazek et al., 1992; Niikura et al., 2001), and IgG or IgM virus-specific antibody capture ELISA (Ksiazek et al., 1999b; Saijo et al., 2001) have been used for the laboratory diagnosis of EBOV infection. As the viral load in the blood reaches extremely high levels in EBOV infection (Fisher-Hoch et al., 1992; Ksiazek et al., 1999a), the detection of EBOV antigens by antigen capture ELISA is suitable as a method of laboratory diagnosis. However, these methods are time-consuming and can require large and expensive apparatus, and are therefore not appropriate for a rapid detection system. Therefore, reverse transcription-PCR (RT-PCR) has taken over as a first choice diagnostic technique for detection of EBOV (Gibb et al., 2001; Leroy et al., 2000; Towner et al., 2004; Weidmann et al., 2004). However, RT-PCR test systems consists of several steps of amplification that require the use of a high-precision thermal cycler.

Loop-mediated isothermal amplification (LAMP) is a novel technique for nucleic acid amplification (Notomi et al., 2000). The use of this method has recently been demonstrated for the diagnosis of several human pathogenic viruses (Parida et al., 2004; Poon et al., 2004). This simple and rapid technique is based on the principle of strand displacing DNA synthesis performed by the *Bst* DNA polymerase large fragment, isothermal conditions at low temperature (60–65 °C), thereby obviating the need for a thermal cycler (Notomi et al., 2000). The technique is highly specific for the target sequence, which is attributable to recognition of the target sequence at six independent sites with four primers. Moreover, the LAMP method generates an increase in turbidity in positive samples allowing detection by visual judgment and by real-time monitoring based on the turbidity of the reaction mixture as well as agarose gel electrophoresis (Mori et al., 2004).

With the goal of developing a rapid, simple, sensitive, and specific molecular test for the diagnosis of EBOV, we developed a ZEBOV-specific RT-LAMP assay.

2. Materials and methods

2.1. Viruses and cells

All virus strains used in this study, ZEBOV strains Mayinga and Zaire 95, SEBOV strains Boniface and Gulu, ICEBOV, and MARV strains Musoke, Ozolin, Ravn, and Angola, were propagated in Vero E6 cells. With the exception of the MARV Angola strain, which was isolated from clinical material from the outbreak in Uige, Angola, all viruses were kindly provided by the Special Pathogens Branch of the Centers for Disease Control (T.G. Ksiazek and P.E. Rollin) and the Virology Division of the US Army medical Research Institute of Infectious Diseases (P.B. Jahrling and T.W. Geisbert). Vero E6 cells (ATCC C1008 CRL1586) were grown in Eagle's minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, kanamycin (60 mg/L), streptomycin (100 mg/L), and penicillin (10^5 U/L) at 37 °C in 5% CO_2.

2.2. Virus infectivity and RNA extraction

Virus infectivity titers (focus-forming units [FFU]) were measured by counting the number of infected cell foci detected by an indirect immunofluorescent antibody assay using previously described procedures (Ebihara et al., 2000). Serial 10-fold-diluted specimens were adsorbed on monolayers of Vero E6 cells grown in 96-well plastic plates for 1 h at 37 °C in 5% CO_2. Cells were then overlaid with growth medium containing 1.5% carboxymethyl cellulose sodium salt (SIGMA, St. Louis, MO). After incubation for 4–5 days at 37 °C in 5% CO_2, the medium was removed, and the cells were washed with PBS and fixed with 10% buffered-PBS formalin. To detect virus antigen, we incubated cells with anti-VP40 rabbit sera, followed by FITC-conjugated anti-rabbit goat IgG antibody (SIGMA).

Viral RNAs were extracted manually from virus suspension with TRIzol (Invitrogen), and resuspended in 100 μL of DEPC-treated water. All infectious materials were handled in the BSL-4 facilities of the National Microbiology Laboratory of the Public Health Agency of Canada.

2.3. Primer design

ZEBOV-specific primers used for RT-LAMP were designed on the basis of the published sequences of strain Mayinga (GenBank Accession no. AF086833). Potential target regions were selected from the sequences aligned with other species of EBOV, and RT-LAMP primers were designed with the LAMP primer design support software program (PrimerExplorer ver.3; Net Laboratory, Tokyo, Japan; http://primerexplorer.jp/e/). We used four primers for the RT-LAMP assay, including two outer primers (F3 and B3), a forward inner primer (FIP), and a reverse inner primer (BIP). The locations and sequences of the primers are shown in Fig. 1A. FIP consisted of F1c complementary to the F1 sequence, a TTTT spacer, and the F2 sequence. BIP consisted of B1c complementary to the B1 sequence, a TTTT spacer, and B2 sequence. F3 and B3 were located outside F2 and B2. The sequences of these primers were compared to an alignment of trailer region sequences of the EBOV species Zaire, Reston, and Sudan, and MARV.

2.4. Preparation of the artificial ZEBOV RNA

Plasmid p3E5EGFP contains the green fluorescent protein (GFP) gene in antisense orientation between the leader and trailer sequences of the ZEBOV (strain Mayinga) genome, flanked by the T7 RNA polymerase promoter and the hepatitis delta virus ribozyme sequence (Watanabe et al., 2004). It was linearized with the restriction enzyme NdeI and then used for in vitro transcription. The artificial ZEBOV RNA was synthesized using the T7 RiboMAX^{TM} expression large-scale RNA production system (Promega, Madison, WI) according to the manufacturer's instructions. The reactions were carried out for