Antibody-Dependent Enhancement of Marburg Virus Infection

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Background. Marburg virus (MARV) and Ebola virus (EBOV) cause severe hemorrhagic fever in primates. Earlier studies demonstrated that antibodies to particular epitopes on the glycoprotein (GP) of EBOV enhanced virus infectivity in vitro.

Methods. To investigate this antibody-dependent enhancement (ADE) in MARV infection, we produced mouse antisera and monoclonal antibodies (mAbs) to the GPs of MARV strains Angola and Musoke.

Results. The infectivity of vesicular stomatitis virus pseudotyped with Angola GP in K562 cells was significantly enhanced in the presence of Angola GP antisera, whereas only minimal ADE activity was seen with Musoke GP antisera. This difference correlated with the percentage of hybridoma clones producing infectivity-enhancing mAbs. Using mAbs to MARV GP, we identified 3 distinct ADE epitopes in the mucinlike region on Angola GP. Interestingly, some of these antibodies bound to both Angola and Musoke GPs but showed significantly higher ADE activity for strain Angola. ADE activity depended on epitopes in the mucinlike region and glycine at amino acid position 547, present in the Angola but absent in the Musoke GP.

Conclusions. These results suggest a possible link between ADE and MARV pathogenicity and provide new insights into the mechanisms underlying ADE entry of filoviruses.

Marburg virus (MARV) and Ebola virus (EBOV) are filamentous, enveloped, negative-strand RNA viruses belonging to the family Filoviridae. These viruses have produced sporadic outbreaks of hemorrhagic fever in human and nonhuman primates [1]. MARV was first identified in 1967 during an outbreak of hemorrhagic fever in Marburg, Germany, and Belgrade, Yugoslavia. These outbreaks were linked to infected monkeys imported from Uganda [2]. Since the first outbreak of Marburg hemorrhagic fever, several sporadic outbreaks have been reported in central African countries [1, 3–6]. The largest outbreak occurred in Uige province in Angola from 2004 to 2005, with a mortality rate of 90% among 252 reported cases [7]. The strain Angola had a higher mortality rate and was thought to be more pathogenic than earlier isolates, such as strain Musoke [8, 9], which was isolated from a human case in 1980 in Kenya [5]. Among EBOV species, *Zaire ebolavirus* seems to be the most virulent, with a case fatality rate of up to 90%; whereas *Reston ebolavirus* has never been associated with symptomatic infection in humans [10] and was shown to be less pathogenic than *Zaire ebolavirus* in nonhuman primates [11].

The filovirus genome encodes at least 7 structural proteins. The fourth gene from the 3' end of the genome encodes the envelope glycoprotein (GP), which undergoes proteolytic cleavage into 2 subunits, GP1 and GP2. The GP1 subunit mediates cell-surface receptor binding [12, 13], and the GP2 subunit is involved in fusion of the viral envelope and host cell membrane [14]. GP is highly glycosylated, with a large amount of N- and O-linked

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glycans, most of which are located in the middle portion of the GP, designated the mucinlike region (MLR) [15, 16]. The amino acid sequences of the MLR are highly variable among filovirus species [17, 18].

It has been demonstrated elsewhere that EBOV infection in humans and nonhuman primates induced GP-specific antibodies that had the ability to enhance viral infectivity of certain cells in vitro [19, 20]. This mechanism, known as antibody-dependent enhancement (ADE) of viral infection, depends mostly on the cross-linking of virus-antibody complexes through interaction with cellular Fc receptors (FcRs) [21]. Our previous studies have identified an additional mechanism underlying EBOV ADE in vitro, namely, complement protein C1q and C1q receptor-dependent ADE [19]. Epitopes involved in ADE were identified predominantly in the MLR of the Zaire EBOV (ZEBOV) GP1 subunit. A possible contribution of ADE to the distinct pathogenicity observed for ZEBOV and Reston EBOV was discussed elsewhere [20, 22]. However, little is known about the role of ADE in MARV pathogenicity.

In this study, we produced mouse antisera and monoclonal antibodies (mAbs) specific to the GPs of MARV strains Angola and Musoke and examined their ADE activities by using vesicular stomatitis virus (VSV) pseudotyped with MARV GPs. We found distinct ADE activity between antibodies to Angola and Musoke GPs, which may be linked to a difference in pathogenicity of these strains. The identified ADE epitopes were all located in the MLR of the GPs, but the presence of these epitopes was not sufficient to give a maximal ADE. Potential mechanisms for effective ADE seen with certain MARV strains are discussed here.

MATERIALS AND METHODS

Viruses and Cells

VSV pseudotyped with Angola GP (VSV-Angola) or Musoke GP (VSV-Musoke) expressing green fluorescent protein was generated as described elsewhere [23]. Deletion mutant GPs, chimeric GPs, and mutant GPs with a single substitution were generated as described elsewhere [24]. There was no significant difference in the infectivity in Vero E6 cells among these viruses, suggesting that the functional GPs were incorporated into VSV virions [24]. To reduce the background infectivity of parent VSV G, the pseudotyped viruses were treated with a neutralizing mAb to VSV G protein (VSV-G[N]1-9) before use. The virus infectivity was determined by counting the number of cells expressing green fluorescent protein, using fluorescence microscopy or flow cytometry. Monkey kidney Vero E6 cells and human embryonic kidney (HEK) 293 and 293T cells were grown in Dulbecco's modified Eagle's medium (Sigma), and human chronic myelogenous leukemia K562 cells bearing FcR were grown in Roswell Park Memorial Institute 1640 medium (Sigma). The media were supplemented with fetal calf serum and antibiotics.

Antisera

To produce antisera to filovirus GPs, 5-week-old female BALB/c mice were immunized subcutaneously twice in a 3-week interval with 100 μg of viruslike particles (VLPs) [25, 26] with complete Freund's adjuvant or intraperitoneally twice in a 3-week interval with 50 μg of VLPs only. The serum samples from intraperitoneally immunized mice were collected 7 days after the second immunization. Subcutaneously immunized mice were boosted intraperitoneally with 100 μg of VLPs alone 3 weeks after the second immunization, and the serum samples were collected 7 days after the boost dose.

Generation of mAbs

Five-week-old female BALB/c mice were immunized subcutaneously with 100 μg of VLPs with complete Freund's adjuvant (Difco). At 3 and 6 weeks after the first immunization, the mice were subcutaneously immunized with 100 μg of VLPs with incomplete Freund's adjuvant (Difco). Three weeks after the last immunization, mice were boosted intraperitoneally twice in a 3-week interval with 100 μg of VLPs only. Three days later, mouse spleen cells and mouse myeloma P3-U1 cells were fused and maintained according to a standard procedure [27]. Hybridomas were screened for secretion of MARV GP-specific mAbs by enzyme-linked immunosorbent assay (ELISA), and hybridoma-producing mAbs were cloned by limiting dilution of the cells. mAbs were purified from mouse ascites using protein A agarose columns (Bio-Rad). The isotypes of the obtained mAbs were determined using a mouse mAb isotyping test kit (AbD Serotec) according to the manufacturer's instructions.

Infectivity Enhancement and Neutralization Tests

Appropriately diluted serum samples or mAbs were mixed with equal volumes of the pseudotyped viruses ($\sim 10^5$ infectious units/mL on Vero E6 cells), followed by 1-hour incubation. Infectivity was then determined in Vero E6, K562, and HEK 293 cells for neutralizing, FcR-, and C1q-dependent ADE activities, respectively, by counting the fluorescent cells, as described elsewhere [19, 20, 22, 23]. The relative percentage of infected cells was determined by setting the number of cells infected in the absence of GP-specific antisera or purified mAbs to 100. Antibodies that gave relative infectivity values of $<50\%$ or $>200\%$ were defined as neutralizing or enhancing antibodies, respectively. For detection of C1q-dependent ADE, viruses were incubated with antisera or mAbs in the presence of C1q (50 μg/mL; Sigma) before infection of HEK 293 cells.

Enzyme-Linked Immunosorbent Assay

ELISA plates (Nunc Maxisorp) were coated with lysate from HEK 293T cells expressing MARV-GP, VLP, or histidine-tagged purified GP, as described elsewhere [28], at 4°C overnight and then washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) before addition of blocking buffer (3% skim milk in PBST) for 2 hours at room temperature. After

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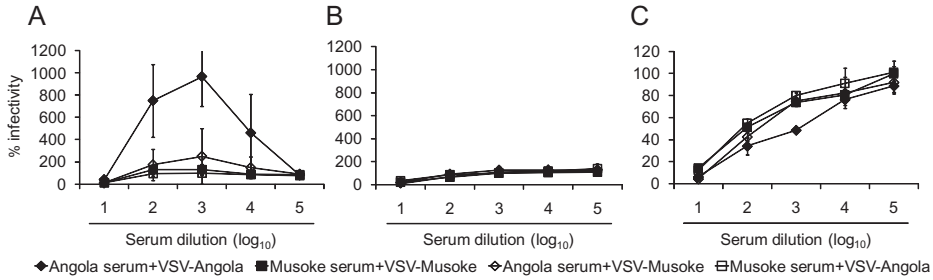

Figure 1. Antibody-dependent enhancement and neutralizing activities of polyclonal antisera from subcutaneously immunized mice. Marburg virus glycoprotein (MARV GP) antisera ($1:10^1$ to $1:10^5$ dilutions) were mixed with vesicular stomatitis virus (VSV) pseudotyped with MARV GPs, incubated for 1 hour at room temperature, and inoculated into 10^5 K562 cells (in 96-well plate) *A*, confluent HEK 293 cells *B*, or confluent Vero E6 cells *C*, Human embryonic kidney 293 cells were infected in the presence of purified C1q (50 μg/mL). Results are expressed as means (± standard deviations) of data for 3 immunized mice. Relative percentages of infected cells are shown as mean values determined based on the number of infected cells in the absence of specific antibodies against MARV GPs (100%).

being washed 3 times with PBST, primary antibodies (ie, hybridoma supernatants, purified mAbs, or antiserum) were added to each well and incubated at room temperature for 1 hour and the plates were washed 3 times with PBST. The antibody binding was detected by goat anti-mouse immunoglobulin (Ig) G1, IgG2a, IgG2b, IgG3 (Bethyl), and IgG (H+L) (Jackson ImmunoResearch) conjugated with horseradish peroxidase. After incubation at room temperature for 1 hour, the plates were washed 4 times with PBST, and 3,3',5,5'-tetramethylbenzidine (Sigma) was added to each well. An equal volume of 1N sulfuric acid was subsequently used to stop the enzyme reaction after 15-minutes incubation, and the optical density value was read at 450 nm on an ELISA plate reader.

Immunostaining

HEK 293T cells were transfected with the mammalian expression plasmid pCAGGS, expressing MARV GPs, and were fixed 48 hours later with methanol for 30 minutes. After blocking with 10% bovine serum albumin in PBS for 90 minutes at room temperature, cells were incubated with mAbs (1 μg/mL) for 1 hour at room temperature. The binding of the mAbs was detected by goat anti-mouse IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch) diluted in 5% bovine serum albumin in PBST. After incubation for 1 hour at room temperature, GP expressed in the cells was visualized with 3,3'-diaminobenzidine.

RESULTS

FcR-Dependent ADE Activity of GP Antisera Differed Between MARV Strains Angola and Musoke

To examine ADE of MARV infection, we first generated antiserum by subcutaneous immunization of mice with MARV strain Angola or Musoke VLPs. The levels of infectivity of VSV-Angola and VSV-Musoke in the presence of these antisera were determined by using K562 and HEK 293 cells for FcR- and C1q-dependent ADE, respectively (Figure 1*A* and 1*B*). In K562 cells, the infectivity of VSV-Angola was markedly enhanced in the presence of Angola GP antiserum at dilutions ranging from $1:10^2$ to $1:10^4$, whereas only minimal enhancement of VSV-Musoke was seen in the presence of Angola GP antiserum. In the presence of Musoke GP antiserum, no apparent enhancement of infectivity was observed for any of the 2 VSV pseudotypes. Furthermore, no C1q-dependent ADE activity in HEK 293 cells was detected in the Angola or Musoke GP antiserum at any dilution (Figure 1*B*), and no significant difference in neutralizing activity was observed between the 2 antisera (Figure 1*C*). Interestingly, cross-reactivity between Angola and Musoke GP antiserum was found in their neutralizing activities but not in FcR-dependent ADE activities.

Difference in C1q-Dependent ADE Activity Between MARV and EBOV Antisera

It was demonstrated elsewhere that intraperitoneal immunization elicited C1q-dependent ADE antibodies to ZEBOV GP more efficiently than subcutaneous immunization [20]. Therefore, we produced MARV strain Angola and Musoke GP antisera by intraperitoneal immunization and compared C1q-dependent ADE activity with ZEBOV GP antisera in HEK 293 cells (Figure 2*A*). We confirmed that ZEBOV GP antisera significantly enhanced the infectivity of VSV pseudotyped with ZEBOV GP in the presence of purified C1q. However, the Angola GP antiserum only minimally enhanced the infectivity of VSV-Angola in the presence of C1q. The Musoke GP antiserum did not show any C1q-dependent ADE activity. Subsequently, we used ELISA to examine the antiserum for the proportion of each IgG subclass (Figure 2*B*). We found that the amount of IgG2a, IgG2b, and IgG3 was significantly lower in the MARV-Angola GP antiserum than in the ZEBOV GP antisera (Student's *t* test, $P < .05$). There was no significant difference in the amount of IgG1 between the MARV-Angola GP and ZEBOV GP antisera.

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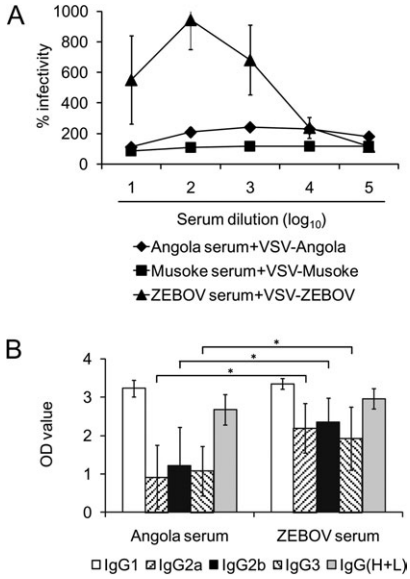

Figure 2. C1q-dependent antibody-dependent enhancement of polyclonal antisera from intraperitoneally immunized mice *A,* and immunoglobulin (Ig) G subclass in the serum samples *B,* Vesicular stomatitis virus (VSV) pseudotyped with Marburg virus glycoproteins (MARV GPs) were incubated with mouse antisera ($1:10^1$ to $1:10^5$ dilutions) in the presence of purified C1q (50 μg/mL) and inoculated into human embryonic kidney 293 cells. Results are expressed as means (± standard deviations) of data for 3 immunized mice. Relative percentages of infected cells are shown as mean values determined based on the number of infected cells in the absence of specific antibodies against MARV GPs (100%). The binding activity of each antibody class in 3 immunized mouse antisera ($1:10^2$) was measured by enzyme-linked immunosorbent assay (ELISA) using histidine-tagged GPs as antigens. Experiments were triplicated and the means and standard deviations were shown. The differences between optical density (OD) values were compared using the Student's *t* test. $*P < .05$ for differences in OD values between *Angola* and *Zaire ebolavirus* (ZEBOV) GPs.

Characterization of mAbs Specific to MARV GPs

We then generated hybridoma cells producing specific mAbs (47 and 28 clones for Angola and Musoke GPs, respectively). Table 1 lists the number of clones and their characteristics (ie, neutralizing or ADE). Though there was no significant difference by the χ^2 test in the percentage of cell clones producing neutralizing antibodies between the Angola- and Musoke-specific clones, the percentage of clones showing FcR-dependent ADE activity was significantly higher after immunization with Angola GP. The clones producing the enhancing antibodies were further classified into 2 groups; the first (group A) consisted of mAbs that enhanced the infectivity of both VSV-Angola and VSV-Musoke, and the second (group B) consisted of mAbs that enhanced only VSV-Angola infectivity. No mAb showed C1q-dependent ADE activity (data not shown).

Identification of ADE Epitopes on MARV GPs

To identify epitopes involved in ADE activity of mAbs, we tested the representative mAbs (IgG1) from groups A and B to determine reactivity to synthetic peptides derived from Angola and Musoke GP (PEPscreen; Sigma), and we found that all mAbs belonging to group A bound strongly to peptides corresponding to amino acid positions 330–350 and 410–430 (data not shown). We then constructed mutant GPs lacking amino acid residues 330–350 (AΔ1) or 410–430 (AΔ2) and examined the reactivity of mAbs of group A (Figure 3). In immunostaining of HEK 293T cells expressing the mutant GPs, these mAbs bound to AΔ1 but not AΔ2, suggesting that the epitopes for these mAbs lay between amino acid positions 410–430 where the sequences are partially shared between Angola and Musoke GPs. By contrast, mAbs belonging to group B failed to bind to any synthetic peptide, suggesting that their epitopes were likely in nonlinear conformation. Because mAbs of group B did not bind to Musoke GP, we constructed chimeric GPs between Angola and Musoke GPs (MA1–MA5), as indicated in Figure 3. Based on the reactivity of mAbs to the chimeric GPs expressed in HEK 293T cells, 2 regions on Angola GP were identified as the epitopes of mAbs of group B (Figure 3). mAbs AGP2-1, AGP40-32, and AGP126-10 reacted strongly with MA1 and MA2 but not MA3, MA4, and MA5, indicating that these mAbs recognized an epitope that resided between amino acid positions 369 and 385 in Angola GP. Another mAb, AGP90-28, bound to 4 chimeric GPs, MA1, MA2, MA3, and MA4, but not MA5, indicating that the epitope for this mAb was located between amino acid positions 402 and 418 in Angola GP. All of these identified epitopes were located within the MLR.

Table 1. Characterization of Monoclonal Antibody (mAb) Clones Specific to Marburg Virus Glycoprotein (GP)

	mAb Clones, no. (%)	
Clone characteristic	Angola GP	Musoke GP
ELISA positive	47 (100)	28 (100)
Neutralizing	19 (40)	9 (32)
Enhancing	15 (32) [a]	0 (0)

NOTE. Differences in the ratios of the clone numbers between Angola and Musoke GPs were analyzed by χ^2 test, and statistical significance was established at $P < .05$. ELISA, enzyme-linked immunosorbent assay.

[a] $P < .05$.

Contribution of GP2 Region to ADE

To confirm the contribution of the MLR of MARV in ADE of these mAbs, we constructed chimeric GPs whose MLRs were swapped between Angola and Musoke GPs (AMA and MAM), and the levels of infectivity of VSV pseudotyped with these chimeric GPs (VSV-AMA and VSV-MAM) were compared in K562 cells (Figure 4). Unexpectedly, however, the infectivity of VSV-MAM was not enhanced by Angola MLR-specific mAbs (ie, mAbs A2-1 and A90-28 in group B). Furthermore, in the presence of mAb AGP127-8 belonging to group A, the infectivity of VSV-AMA was still significantly higher than that of

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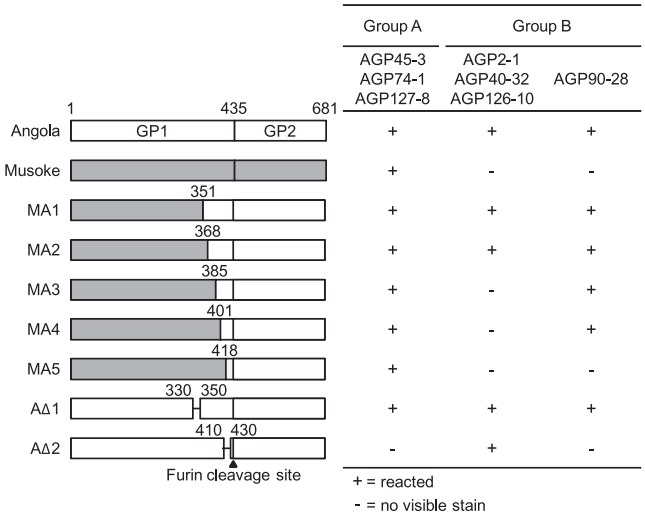

Figure 3. Reactivity of monoclonal antibodies to chimeric and deletion mutant glycoproteins (GPs). Reactivity was tested by immunostaining, as described in Materials and Methods. Numbers indicate amino acid positions of the GP primary amino acid sequence.

VSV-Musoke, and replacement of the MLR of Musoke GP with that of Angola GP (VSV-MAM) had little effect on the enhancement of infectivity compared with the infectivity of VSV-Musoke. We then examined the infectivity of VSV pseudotyped with a series of chimeric GP mutants, as shown in Figure 4, and found that only the infectivity of VSV-MAA, which had the MLR and part of GP2 (shown as GP2C in Figure 4) derived from Angola GP, was reasonably enhanced by mAbs of both groups A and B. These results indicated that both the MLR and the GP2 region of Angola GP were required for maximal ADE activity of these mAbs.

Importance of an Amino Acid at Position 547 for ADE

The GP2C regions of the Angola and Musoke GPs differ in 4 amino acids: positions 504, 547, 596, and 618. To identify which amino acid in GP2C contributed to the differential infectivity enhancement by mAbs AGP127–8, A2-1, and A90-28, the following 8 mutant GPs that contained single-amino-acid substitutions were constructed: 4 Angola-based mutant GPs (A/H504T, A/G547V, A/A596T, and A/R618K) and 4 Musoke-based mutant GPs (M/T504H, M/V547G, M/T596A, and M/K618R) (Figure 5). The infectivity of VSV pseudotyped with these mutant GPs in the presence of these mAbs was compared in K562 cells. We found that the substitution at position 547 clearly switched the viral infectivity in the presence of mAb AGP127-8, as shown by substantially enhanced and reduced infectivity of VSV-M/V547G and A/G547V, respectively, when compared with VSV pseudotyped with wild-type Musoke and Angola GPs. By contrast, the mutations at position 504, 596, and 618 had limited effects on the infectivity of the respective viruses. Interestingly, ADE of VSV-A/G547V infectivity was not observed, even in the presence of Angola GP-specific mAbs, AGP2-1 and AGP90-28. These results indicated that amino acid position 547 played an important role in the ADE activities of these mAbs. To examine whether reduced ADE activities of mAbs resulted from the loss of binding affinity due to the structural change by the mutation at position 547, we measured reactivity to each GP with ELISA (Figure 6). We found that Angola and Musoke GP antisera reacted similarly to all the GP

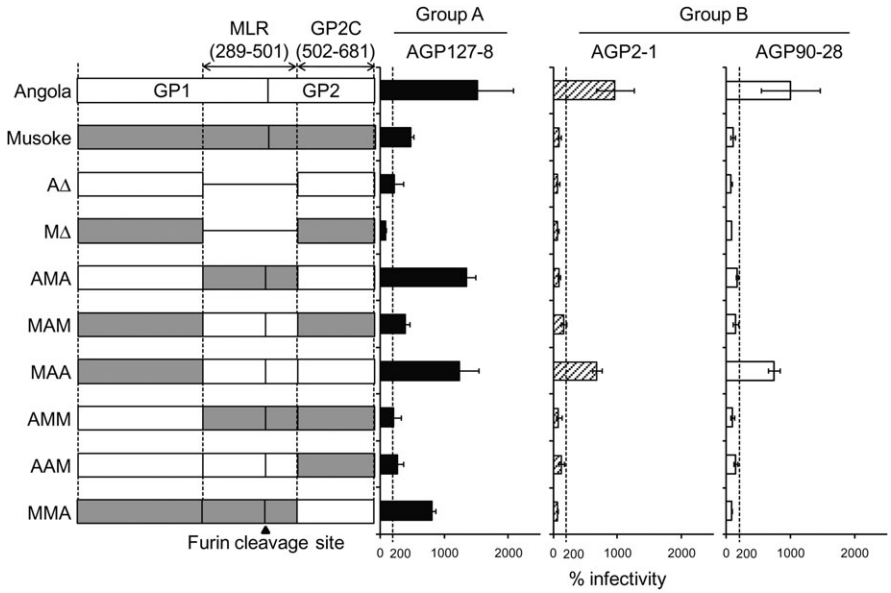

Figure 4. Infectivity of vesicular stomatitis virus pseudotyped with deletion or chimeric mutant glycoproteins (GPs) in K562 cells. VSV pseudotyped with mutant GPs was incubated with monoclonal antibodies AGP127-8, AGP2-1, and AGP90-28 (1 μg/mL) and then inoculated into K562 cells. All experiments were done in triplicate, and means and standard deviations are shown. Numbers indicate amino acid positions of the GP primary amino acid sequence.

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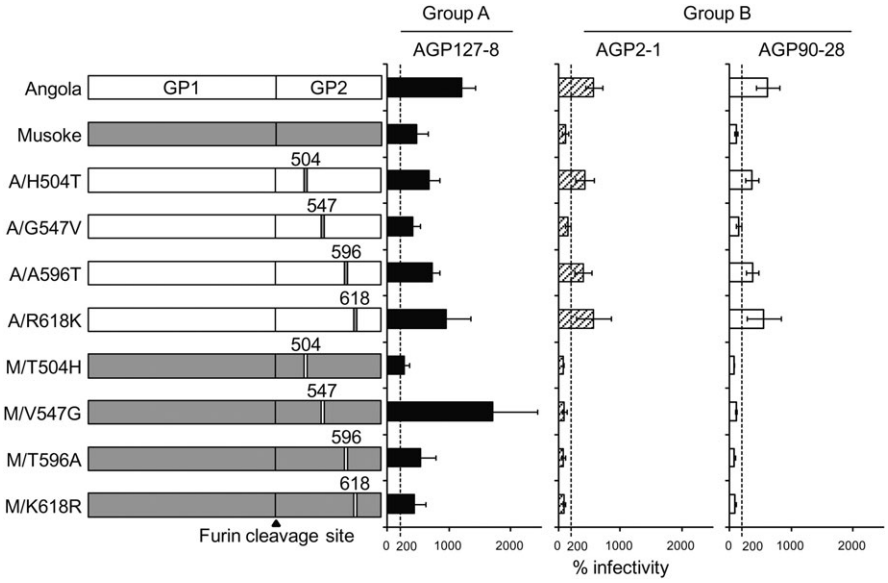

Figure 5. Infectivity of vesicular stomatitis virus pseudotyped with the mutant glycoproteins (GPs) with a single substitution in K562 cells. Experimental conditions were the same as described for Figures 1 and 4. All experiments were done in triplicate, and means and standard deviations are shown.

antigens, suggesting that the substitution at position 547 did not change the overall antigenic structure of MARV GPs. Importantly, this substitution resulted in no significant change in reactivity of these mAbs, indicating that the substitution at position 547 did not affect the binding affinity of these mAbs to GPs.

DISCUSSION

It has been shown that in addition to the common receptor- or coreceptor-dependent mechanism of cellular attachment, some viruses use antiviral antibodies for efficient entry into target cells [21]. Although this phenomenon, ADE of EBOV, has been

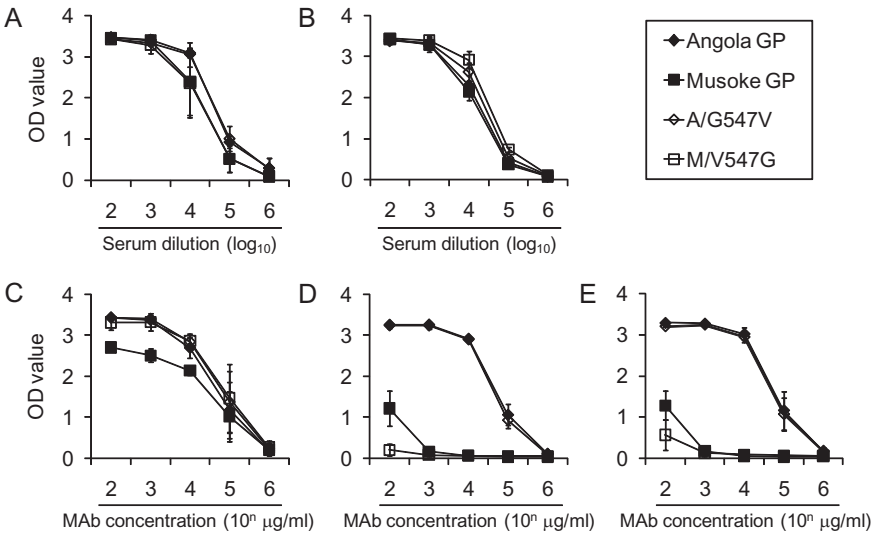

Figure 6. Binding affinities of the mouse antisera and monoclonal antibodies to Marburg virus glycoproteins (MARV GPs). Serial 10-fold dilutions of the mouse antisera to Angola GP *A* and Musoke GP *B* or indicated concentrations of mAbs AGP127-8 *C*, AGP 2-1 *D*, and AGP 90-28 *E*, were tested by enzyme-linked immunosorbent assay (ELISA) for reactivity to wild-type GPs and GPs with single amino acid substitutions. Viruslike particles expressing each GP were used as ELISA antigens. Each optical density (OD) value represents the means and standard deviations of 2 independent experiments.

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studied by using mouse mAbs, convalescent human, and experimentally infected nonhuman primate serum samples [19, 20, 22], the possible contribution of MARV-specific antibodies to ADE has not been reported previously to our knowledge.

In the present study, we demonstrated that the infectivity of VSV-Angola in K562 cells was enhanced notably in the presence of Angola GP antisera (ie, FcR-dependent ADE), whereas Musoke GP antisera did not significantly enhance the infectivity of VSV-Angola or -Musoke (Figure 1). This difference between the 2 MARV strains was also supported by the observation that immunization with Angola GP induced significantly higher numbers of B-cell clones with infectivity-enhancing properties than did immunization with Musoke GP (Table 1). These results may suggest that the potential difference in the pathogenicity between the MARV strains Angola and Musoke might be partially explained by the ability to induce infectivity-enhancing antibodies, as was proposed for the distinct pathogenicity seen with ZEBOV and Reston EBOV [20, 22].

However, it has been argued whether the strain Angola is more virulent for humans and nonhuman primates than the strain Musoke. Along with the high case fatality rate in the Angola epidemic, it was also noted that in most earlier outbreaks of Marburg hemorrhagic fever, the case fatality rates did not exceed 50% [2, 4, 5], but there was another outbreak in which the case fatality rate was 83%, quite similar to what was seen in the Angola outbreak [29]. In experimental infection of macaques, 3 animals infected with the strain Angola died after illness that was more rapidly progressive than those caused by other viruses tested in other experiments [8, 9, 30], and necropsy showed infection of the liver with accompanying necrosis. However, because animals infected with the strains Angola and Musoke have not been compared in the same experiment, it could only be concluded that infection with the strain Angola ''appeared to progress more rapidly'' than other MARVs tested in other studies. In addition, one of the patients infected with the strain Musoke developed a rapidly progressive illness that closely resembled the features of illness seen in macaques experimentally infected with the strain Angola [5, 9]. Thus, although our results may suggest the possible contribution of ADE to differences in pathogenicity between the strains Angola and Musoke, further investigations are needed to conclude that the strain Angola has a uniquely higher pathogenicity.

Compared with ZEBOV GP antisera, Angola GP antisera enhanced the infectivity of VSV-Angola less efficiently in the presence of C1q (Figure 2*A*). We therefore compared the profile of IgG subclasses in the antisera. A significant difference was seen in the levels of IgG2a, IgG2b, and IgG3 between MARV Angola and ZEBOV GP antisera (Figure 2*B*). It is likely that IgG2a and IgG3 play a more prominent role in C1q-dependent ADE activity, because these classes of antibodies are thought to have a higher affinity for C1q molecules than IgG1 [20]. Thus, this difference may account for the lower activity of C1q-dependent ADE in Angola antisera. It will be of interest to elucidate why MARV and EBOV GPs induce a distinct antibody repertoire.

It was shown that most of the ADE epitopes of EBOV GP were located in the MLR [20]. In the present study, we identified 3 distinct ADE epitopes on MARV GP, and these ADE epitopes were mostly located in the MLR of the GP1 subunit (Figure 3). To further ascertain the contribution of the MLR of MARV GP to ADE, we examined the infectivity of VSV pseudotyped with the Angola GP lacking the MLR in K562 cells in the presence of the Angola GP antisera and found that ADE activity was reduced drastically (data not shown). These results suggested that the MLR of MARV GP also contained dominant epitopes for antibodies that enhance viral infectivity.

Two ADE epitopes, amino acid positions 369–385 and 402–418 (group B epitopes), were recognized by Angola GP-specific mAbs, whereas another epitope, amino acids 410–430, was shared between the 2 GPs (group A epitope). Accordingly, mAbs belonging to group A bound to both Angola and Musoke GP and enhanced the infectivity of both VSV-Angola and -Musoke (Figures 4 and 5). These results suggest that Angola and Musoke GPs share some epitopes recognized by the ADE antibodies. However, Musoke GP antisera and the Musoke GP-specific mAbs did not show ADE activity, suggesting that Musoke GP contained fewer ADE epitopes than Angola GP. Thus, we propose that the primary amino acid structure and glycosylation pattern of the MLR may influence epitope exposure on the GP molecule.

It was noted that the substitution at amino acid position 547 in the GP2 region affected ADE significantly but did not change the binding affinity of the mAbs to the Angola and Musoke GPs (Figure 6). Amino acid position 547 is located at the base of the internal fusion loop [24]. We demonstrated elsewhere that a single-amino acid substitution at position 547 affected the efficiency of C-type lectin-mediated entry and the mechanism underlying endosomal entry such as proteolytic processing by endosomal cathepsin [24]. The present findings also suggest that the amino acid at position 547 plays a role in entry mediated through the ADE pathway. When virus particles are internalized in endosomes via the ADE pathway, it is possible that (1) glycine at position 547 weakens the interaction between GP1 and GP2, resulting in increased cathepsin susceptibility or reduced cathepsin dependence in endosomes and (2) the amino acid at position 547 affects the flexibility of the fusion loop and/or the conformational change needed for fusion. It would be interesting to investigate the role of this particular amino acid for proper GP functions.

In this study, we demonstrated in vitro ADE for MARV that might be associated with the distinct pathogenicity of certain MARV strains. The ADE epitopes were localized in the MLR of GP. Furthermore, our data suggest that the efficiency of ADE-mediated entry of MARV is controlled by the epitope structure on GP and the mechanisms underlying endosomal

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entry. Further in vitro studies are required to prove the contribution of ADE to the exacerbation of MARV infection; these studies should use serum samples from infected monkeys and patients to confirm whether ADE is induced by actual MARV infection and include animal experiments using a reverse-genetics system to introduce mutations into the ADE epitopes and reduce the production of ADE antibodies. The present study, however, together with our previous study [24], provides new insights into the molecular basis of MARV entry, mediated by interaction between the GP MLR and cellular attachment factors, such as C-type lectins and MLR-specific antibodies.

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