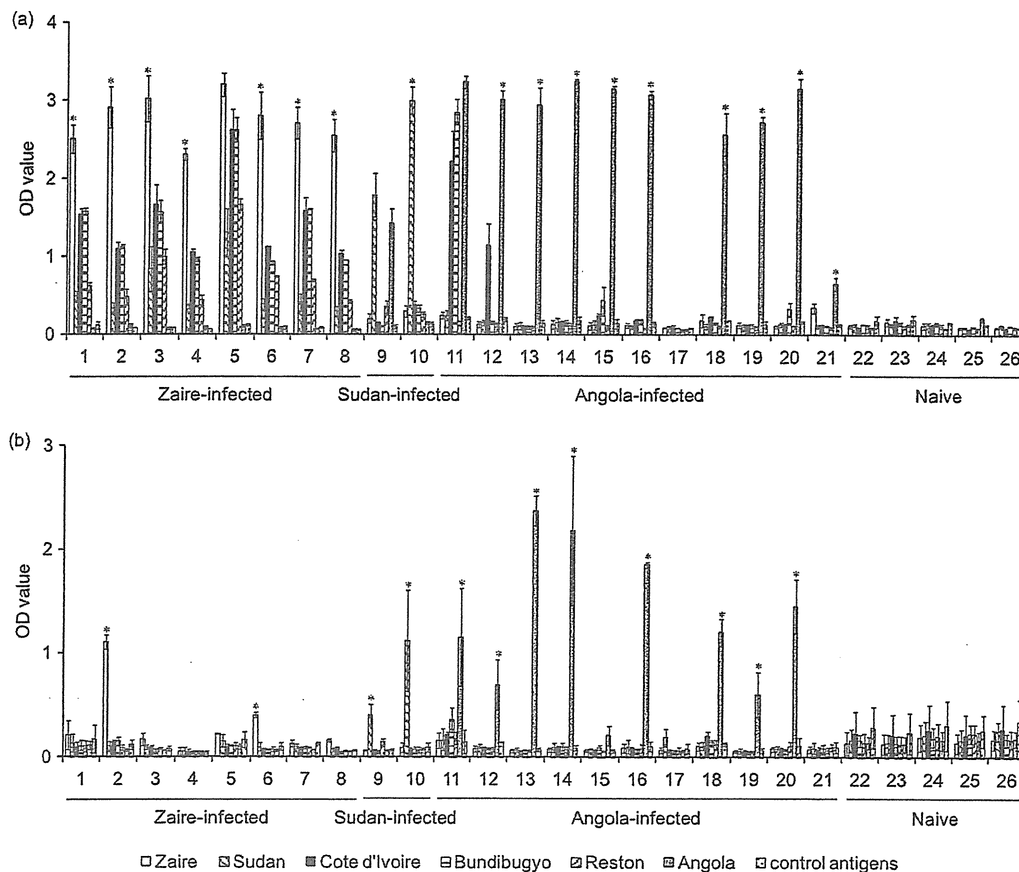

FIG. 6. IgG and IgM antibodies detected in human samples. OD values for specific IgG (a) and IgM (b) antibodies in the patient sera are shown. Sera from 21 individuals were analyzed at 1:1,000 dilutions. Naïve human sera (1:100 dilution) were used as a negative control. Each bar represents the average and standard deviation of data from independent experiments. Asterisks indicate statistically significant differences in OD values between the homologous antigen and all other antigens ($P < 0.05$).

epitopes in the variable regions of the protein. Expectedly, the serological classification mirrors the phylogenetic relationship of the different GPs (Fig. 1). Interestingly, serological characterization of anti-BEBOV antibodies clearly supports the molecular investigations (31) suggesting that BEBOV represents a new species within the EBOV genus.

IgG antibodies in some of the serum and plasma samples collected from infected monkeys and humans showed appreciable cross-reactivity to heterologous antigens, whereas antibodies in the mouse sera produced by immunization with VLPs specifically reacted to the homologous antigens. This result led us to the conjecture that VLP immunization and live-virus infection induce a distinct antibody repertoire or that the antibody repertoire of mice differs from that of primates. Interestingly, the plasma of patient 11 infected with MARV Angola contained IgG, but not IgM, antibodies cross-reactive to His-CIEBOV-GP and His-BEBOV-GP. It might be possible that prior to infection with MARV Angola, this patient was infected with CIEBOV, BEBOV, or another unknown filovirus whose GP has epitopes shared among CIEBOV and BEBOV. In the plasma of patient 17, neither IgG nor IgM antibodies were readily detected. An explanation for this observation might be differences of immunological conditions in individuals, or alternatively, the blood samples have been collected before a detectable antibody response was induced.

Notably, our GP-based ELISA detected MARV Angola-specific IgM antibodies in most of the plasma samples collected during the acute or subacute phase of infection, although it was reported previously that the detection of antibodies is of only limited use for acute-case diagnosis due to a lack of a detectable antibody response (8). The present study suggests that if proper antigen and sensitive assays are available, IgM antibodies can be useful for the diagnosis of acute EBOV and MARV infections and support the use of antigen capture ELISA and reverse transcription-PCR, the most commonly used technologies.

Despite the more recent discovery of REBOV in domestic pigs in the Philippines (1) and the discovery of fruit bat species as potential reservoirs for EBOV and MARV (9, 15, 30, 32), the search for the reservoirs and potential amplifying hosts remains ongoing. Advanced diagnostic technologies are welcome here, and our new GP-based species-specific antibody detection ELISA may be a useful tool for future ecological and seroepidemiological studies in areas of Central Africa and parts of Asia where the disease is endemic.

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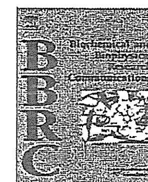

C-type lectins do not act as functional receptors for filovirus entry into cells

Keita Matsuno [a], Eri Nakayama [a], Osamu Noyori [a], Andrea Marzi [b], Hideki Ebihara [b], Tatsuro Irimura [c], Heinz Feldmann [b], Ayato Takada [a,*]

[a] *Department of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan*
[b] *Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rocky Mountain Laboratories, Hamilton, MT, USA*
[c] *Graduate School of Pharmaceutical Science, University of Tokyo, Tokyo, Japan*

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ABSTRACT

Cellular C-type lectins have been reported to facilitate filovirus infection by binding to glycans on filovirus glycoprotein (GP). However, it is not clearly known whether interaction between C-type lectins and GP mediates all the steps of virus entry (i.e., attachment, internalization, and membrane fusion). In this study, we generated vesicular stomatitis viruses pseudotyped with mutant GPs that have impaired structures of the putative receptor binding regions and thus reduced ability to infect the monkey kidney cells that are routinely used for virus propagation. We found that infectivities of viruses with the mutant GPs dropped in C-type lectin-expressing cells, parallel with those in the monkey kidney cells, whereas binding activities of these GPs to the C-type lectins were not correlated with the reduced infectivities. These results suggest that C-type lectin-mediated entry of filoviruses requires other cellular molecule(s) that may be involved in virion internalization or membrane fusion.

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1. Introduction

Ebola virus (EBOV) and Marburg virus (MARV) are enveloped negative-strand RNA viruses that constitute the family *Filoviridae*. Filovirus infection causes severe hemorrhagic fever in humans and non-human primates and mortality rates have ranged up to 90%. *Zaire ebolavirus* (ZEBOV) has caused multiple large outbreaks with the highest mortality rates (~90%) among EBOV species. Among MARVs, strain Angola (MARV-A) caused the largest outbreak in 2004–05 in Angola, with the highest mortality rate (90%) [1].

It has been shown that the filovirus entry into host cells depends on endosomal acidification [2,3] and proteolysis of the glycoprotein (GP) by endosomal cysteine proteases like cathepsin B and/or L [4]. Filovirus GP is the only spike protein on the surface of the virion, and therefore GP is responsible for both receptor binding and membrane fusion. GP is comprised of two molecules, GP1 and GP2, which are linked by a disulfide bond. GP1 contains a putative receptor binding region (RBR) [5,6] and a mucin-like region (MLR) that has a number of potential N- and O-linked glycosylation sites [7,8]. GP2 has a transmembrane domain, cytoplasminc tail and an internal fusion loop [1].

GP1, in particular MLR, is highly glycosylated by both N- and O-glycans, and these glycans are thought to be recognized by cellular C-type lectins such as liver-specific C-type lectin asialoglycoprotein receptor (ASGP-R) [9,10], dendritic cell- and liver/lymph node-specific ICAM-3-grabbing nonintegrin (DC-SIGN and L-SIGN) [10–18], human macrophage galactose-type C-type lectin (hMGL) [18,19], and liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin) [12,17]. Though these C-type lectins show different specificities, depending on the structures of target glycans, all have been reported to promote filovirus entry. Hepatocytes, dendritic cells, monocytes and macrophages are thought to be the preferred target cells of filoviruses, and infection of these cells is important for hemorrhagic manifestation and immune disorders [20–23]. Thus, increased infection of these cells might be directly involved in the pathogenesis of filovirus infection [18,24].

Though the C-type lectins have been reported to enhance filovirus infection, DC-SIGN and L-SIGN did not confer susceptibility for EBOV to non-susceptible cells, i.e. CD4+T-cells [11] and Ramons B cells [14]. In readily susceptible cells, it was reported that the internalization of DC-SIGN and L-SIGN themselves was not essential for trafficking EBOV into endosomal compartments [14]. These studies suggest that C-type lectins promote the filovirus entry by enhancing the virion attachment on the cell surface but not by enhancing the virion internalization. However, it has not been clarified yet whether C-type lectins independently act as a functional receptor mediating all the steps of viral entry including attachment, internalization, and membrane fusion. In the present study, to confirm the role of the C-type lectins in filovirus entry, we generated mutant GPs whose RBRs were impaired, and examined their abilities

* Corresponding author. Fax: +81 11 706 7310.
E-mail address: atakada@czc.hokudai.ac.jp (A. Takada).

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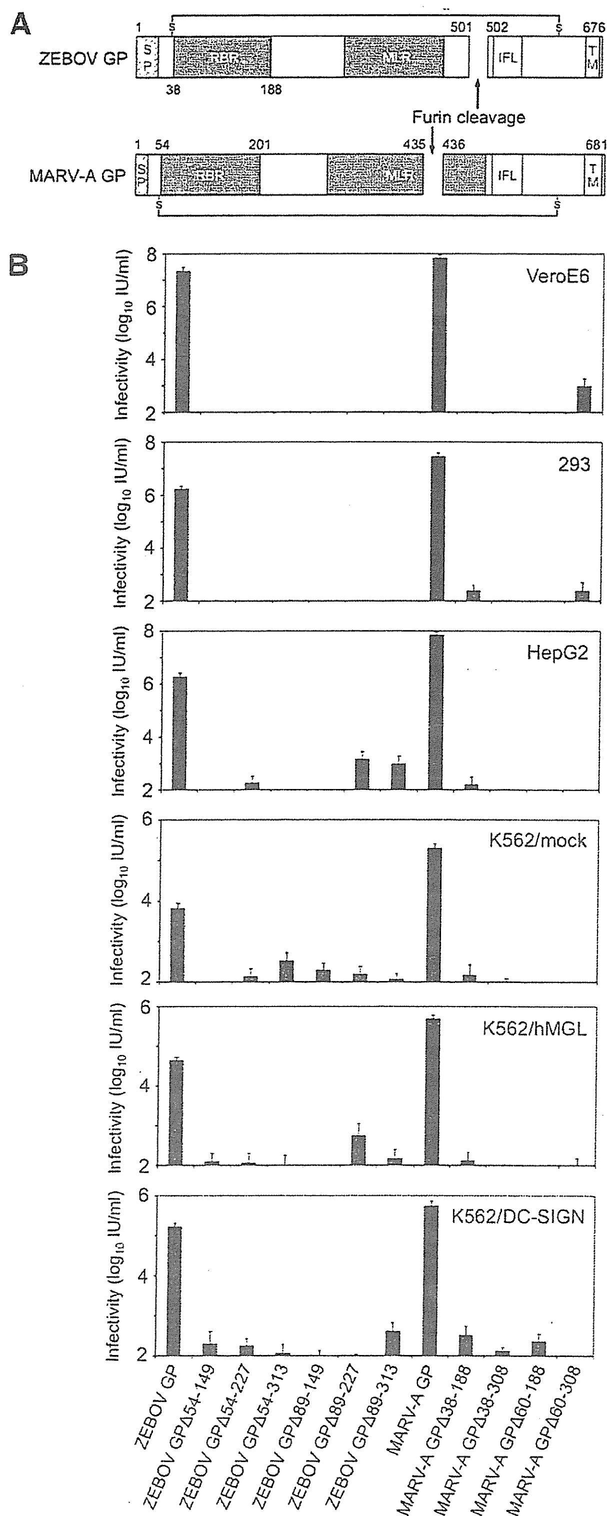

Fig. 1. Infectivity of VSVΔG* pseudotyped with GPΔRBR. Functional domains and putative regions of ZEBOV GP and A-MARV GP are represented in schematic forms (A) (SP; signal peptide, RBR; receptor binding region, MLR; mucin-like region, IFL; internal fusion loop, and TM; transmembrane domain). Infectivities of the viruses in Vero E6, 293, HepG2, K562/mock, K562/hMGL, and K562/DC-SIGN were determined by counting GFP-positive cells and the infectious units (IUs) are indicated on the vertical lines (B). All experiments were done at least three times and averages and standard deviations are shown.

to infect C-type lectin-expressing cells without the interaction between RBR and its unknown putative counterpart(s).

2. Materials and methods

2.1. Cells

293T, Vero E6, and HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics. HepG2 cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics. K562 cell clones expressing hMGL (K562/hMGL), DC-SIGN (K562/DC-SIGN), and mock transfected (K562/mock) were grown in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics.

2.2. Viruses

Construction of mutant GPs was done as previously described [18]. The modified GP genes were then ligated into pCAGGS and used to express GPs on 293T cells. Vesicular stomatitis virus expressing green fluorescent protein (GFP) (VSVΔG*) pseudotyped with GP was generated in 293T cells as previously described [2,18].

Table 1
Characteristics of entry deficient mutant GPs.

	Protein expression[a]	Virion incorporation[b]	Reference
ZEBOV GP	++++	++++	
ZEBOV GPΔ54-149	++	+	
ZEBOV GPΔ54-227	++++	+++	
ZEBOV GPΔ54-313	ND	ND	
ZEBOV GPΔ89-149	++	+	
ZEBOV GPΔ89-227	++++	++++	
ZEBOV GPΔ89-313	ND	ND	
D55A	++++	++++	[27]
L57A	++++	++++	[27]
L57I	++++	++++	[27]
L57F	++++	++++	[27]
L57K	++++	++++	[27]
L63A	++++	+++	[27]
R64E	++++	++++	[27]
F88A	++++	++	[27,28]
K95A	++++	+++	[27]
R134A	++++	++	[29]
K140A	++++	+++	[29]
G143A	++++	+++	[29]
I170A	++++	+++	[27]
MARV-A GP	++++	++++	
MARV-A GPΔ38-188	+	++++	
MARV-A GPΔ38-308	ND	ND	
MARV-A GPΔ60-188	+	++++	
MARV-A GPΔ60-308	ND	ND	
L41A	++++	++++	
K79A	++++	++++	
K118A	++++	++++	
G127A	++++	++++	
Y146A	ND	ND	

++++: >75% of wild-type GP.
+++: 50–75% of wild-type GP.
++: 25–50% of wild-type GP.
+: <25% of wild-type GP.
ND: GP specific bands not detected.
[a] Intensities of GP specific bands in the lysate of 293T cells.
[b] The ratio between intensities of GP and the VSV M specific band in the supernatant of 293T cells.

2.3. Western blot analysis

Anti-ZEBOV GP monoclonal antibody (MAb) 42/3.7 recognizing a linear epitope (amino acid positions 286–296) of ZEBOV GP [25], anti-MARV-A GP MAb 127-8 recognizing a linear epitope (amino acid positions 410–430) of MARV-A GP [25], and anti-VSV matrix protein (M) MAb 192/1 [18] were used for detection of the proteins. Peroxidase-conjugated AffiPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch) and Immobilon Western (Millipore) were used for visualization of the protein bands. Intensities of specific bands were measured with ImageJ [26].

2.4. Lectin-binding assay

VSVΔG* pseudotyped with GPs was purified by ultracentrifugation through a 25% sucrose cushion and diluted in phosphate-buffered saline (PBS). The GP amounts in the VLPs were quantified by Western blotting using MAb ZGP42/3.7 or AGP127-8, and standardized based on the band intensities. Enzyme-linked immunosorbent assay (ELISA) plates were coated with the diluted viruses (2 mg/ml) and then blocked with 3% bovine serum albumin in PBS. After each well was washed with Dulbecco's Tris-buffered saline (dTBS), biotinylated soluble recombinant hMGL (hMGL ECD) or DC-SIGN (DC-SIGN ECD) [18] in dTBS was added. To detect C-type lectins bound to the viruses, horseradish peroxidase (HRP)-streptavidin (Jackson ImmunoResearch) and 3,3,5,5-tetramethylbenzidine (Sigma) were used.

3. Results and discussion

We first constructed RBR-deletion mutant GPs of ZEBOV GP (Δ54-149, Δ54-227, Δ54-313, Δ89-149, Δ89-227, and Δ89-313) and MARV-A GP (Δ38-188, Δ38-308, Δ60-188, and Δ60-308) (Fig. 1A), and viruses pseudotyped with these mutant GPs were generated. Lysates of GP-expressing 293T cells and culture supernatants containing pseudotyped viruses were examined by SDS-PAGE and Western blot analysis to verify the expression and the virion incorporation of the GPs (Table 1). Though MAbs 42/3.7 and 127-8 failed to react with ZEBOV GPΔ54-313, ZEBOV GPΔ89-313, MARV-A GPΔ38-308, and MARV-A GPΔ60-308, the other mutant GPs were detected by these antibodies. Although ZEBOV GPΔ54-149, ZEBOV GPΔ89-149, MARV-A GPΔ38-188, and MARV-A GPΔ60-188 showed significantly lower band intensities than wild-type GP, the expression on 293T cells and incorporation into the virion of these mutant GPs were verified. We then tested the infectivity of VSVΔG* pseudotyped with GPs in the various cell lines (Fig. 1B). The infectivity of VSVΔG* bearing the RBR-deletion mutant GPs was undetectable or significantly lower than VSVΔG* bearing wild-type GPs in all the cells tested, including the C-type lectin-expressing cells. These results indicated that GPs lacking

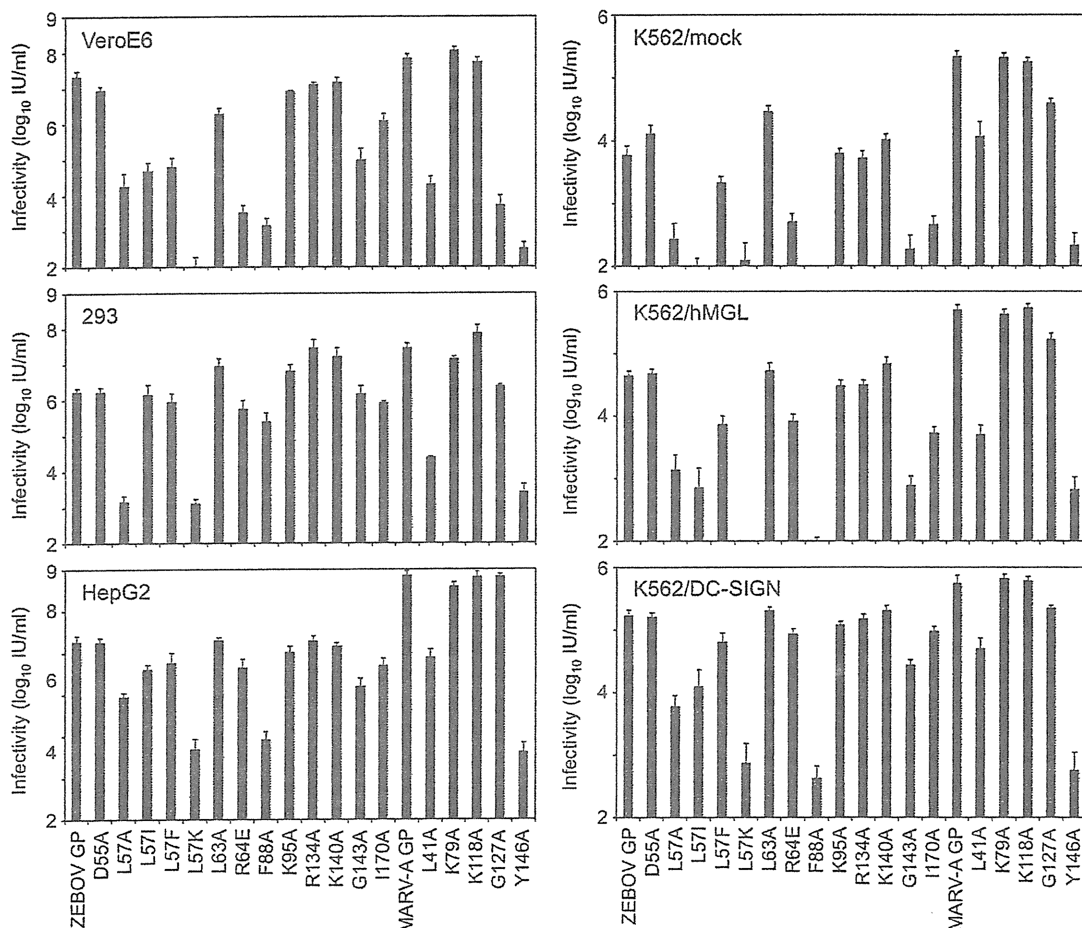

Fig. 2. Infectivity of VSVΔG* pseudotyped with mutant GPs having single amino acid substitutions. The infectious units determined for each virus in Vero E6, 293, HepG2, K562/mock, K562/hMGL, and K562/DC-SIGN are indicated on the vertical lines. All experiments were done at least three times and averages and standard deviations are shown.

RBR did not confer the sufficient infectivity to VSVΔG*, even when the C-type lectins existed on the target cell surface.

However, deletion of entire RBR polypeptides might cause not only a defect of binding ability to the putative functional receptor but also defects in other essential functions such as membrane fusion. Therefore we constructed mutant ZEBOV GPs with single amino acid substitutions in RBR, which were reported to impact the receptor binding capacity, leading to reduced infectivity [27–29]. Based on the amino acid sequence alignment between ZEBOV and MARV-A GPs, MARV-A mutant GPs that had corresponding mutations were also constructed (Table 1). The expression and virion incorporation of each mutant GP were compared with those of wild-type GPs by Western blot analysis (Table 1). Consistent with previous studies [27–29], all the mutant GPs were expressed and incorporated into the virion except MARV-A GP Y146A. The infectivity of the VSVΔG* pseudotyped with mutant GPs was tested in the same cell lines used in Fig. 1 (Fig. 2). As expected, almost all mutant ZEBOV GPs conferred lower infectivity to VSVΔG* in Vero E6, 293, and K562/mock cells than wild-type ZEBOV GP. Similarly, mutations in MARV-A GP (L41A and G127A) significantly reduced the infectivities of the viruses. In the C-type lectin-expressing cells (HepG2, K562/hMGL, and K562/DC-SIGN), the infectivities of the viruses bearing the mutant GPs were also lower than those of the viruses with wild-type GPs, and were likely reduced parallel to the infectivities in Vero E6, 293, and K562/mock cells. These results suggested that the reduced infectivity caused by the mutations in RBR could not be complemented by the interaction between the glycans on GP and C-type lectins.

In a lectin-binding assay using pseudotyped viruses and soluble recombinant hMGL (hMGL ECD) and DC-SIGN (DC-SIGN ECD), we further confirmed that the binding capacities of GPs to these lectins were not significantly reduced by the mutations that gave the lowest infectivities to VSVΔG* in K562/hMGL and K562/DC-SIGN (i.e., F88A and L41A of ZEBOV and MARV-A GPs, respectively) (Fig. 3). This finding indicated that there was no remarkable correlation between GP binding capacity to C-type lectins and reduced infectivity of the viruses with the mutant GPs, and suggested a limited contribution of the interaction between C-type lectin and GP to the subsequent steps in filovirus entry.

In the present study, we demonstrated that the structure of RBR was essential for the entry of filoviruses even when C-type lectins existed on the cell surface, suggesting that the C-type lectins were not independently able to mediate filovirus entry into cells. Therefore, we conclude that C-type lectin-mediated entry of filoviruses requires other cellular molecule(s) that may be critical for virion internalization and/or membrane fusion. Identification of the unknown ubiquitous receptor(s) or coreceptor(s) is essential for further understanding of the molecular mechanisms of filovirus cellular entry and may provide information on the link to the tropism and pathogenesis of filovirus infection.

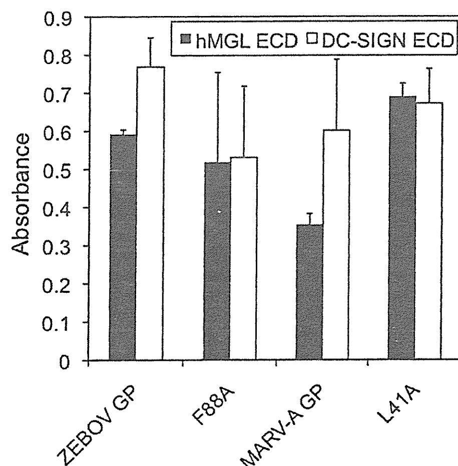

Fig. 3. Binding capacity of the C-type lectins to VSVΔG* pseudotyped with MARV GPs. ELISA plates were coated with purified VSVΔG* bearing mutant GPs. Biotinylated recombinant soluble hMGL ECD (2.5 mg/ml) and DC-SIGN ECD (2.5 mg/ml) were incubated with the viruses and visualized as described in Materials and Methods. All experiments were done in triplicate, and average results and standard deviations are shown.

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Different Potential of C-Type Lectin-Mediated Entry between Marburg Virus Strains[∇]

Keita Matsuno,[1] Noriko Kishida,[2] Katsuaki Usami,[3] Manabu Igarashi,[1] Reiko Yoshida,[1] Eri Nakayama,[1] Masayuki Shimojima,[4] Heinz Feldmann,[5]† Tatsuro Irimura,[3] Yoshihiro Kawaoka,[4] and Ayato Takada[1]*

Department of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan[1]; Laboratory of Influenza Virus Surveillance, Center for Influenza Virus Research, National Institute of Infectious Diseases, Tokyo, Japan[2]; Graduate School of Pharmaceutical Science, University of Tokyo, Tokyo, Japan[3]; Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan[4]; and Special Pathogens Program, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba, Canada[5]

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The glycoproteins (GPs) of filoviruses are responsible for virus entry into cells. It is known that GP interacts with cellular C-type lectins for virus attachment to cells. Since primary target cells of filoviruses express C-type lectins, C-type lectin-mediated entry is thought to be a possible determinant of virus tropism and pathogenesis. We compared the efficiency of C-type lectin-mediated entry between Marburg virus strains Angola and Musoke by using a vesicular stomatitis virus (VSV) pseudotype system. VSV pseudotyped with Angola GP (VSV-Angola) infected K562 cells expressing the C-type lectin, human macrophage galactose-type C-type lectin (hMGL), or dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) more efficiently than VSV pseudotyped with Musoke GP (VSV-Musoke). Unexpectedly, the binding affinity of the C-type lectins to the carbohydrates on GPs did not correlate with the different efficiency of C-type lectin-mediated entry. Site-directed mutagenesis identified the amino acid at position 547, which switched the efficiency of C-type lectin-mediated entry. In a three-dimensional model of GP, this amino acid was in close proximity to the putative site of cathepsin processing. Interestingly, the cathepsin inhibitors reduced the infectivity of VSV-Angola less efficiently than that of VSV-Musoke in C-type lectin-expressing K562 cells, whereas only a limited difference was found in control cells. The amino acid at position 547 was critical for the different effects of the inhibitors on the virus infectivities. These results suggest that the efficiency of C-type lectin-mediated entry of filoviruses is controlled not only by binding affinity between C-type lectins and GP but also by mechanisms underlying endosomal entry, such as proteolytic processing by the cathepsins.

Marburg virus (MARV) and Ebola virus (EBOV), which belong to the family *Filoviridae*, have produced sporadic outbreaks of hemorrhagic fever in Africa. After the initial outbreak of MARV infection in 1967 in Europe, which resulted in 7 deaths among 32 confirmed patients (41), there were three small, isolated outbreaks of MARV infection in Africa between 1975 and 1987. During one of the outbreaks in Kenya in 1980, one of the two patients died (42), and experimental studies showed that this Kenyan MARV Musoke strain (Musoke) killed monkeys within 12 days after infection (6). On the other hand, throughout a recent outbreak of MARV infection in Angola, 84% of the 422 patients died (29). This MARV Angola strain (Angola) produced fatal disease in monkeys within 8 days after inoculation and was thought to be more pathogenic than the Musoke strain (5, 17). Among EBOVs, a difference in pathogenicity was also suggested. Zaire EBOV is thought to be the most pathogenic EBOV, killing approximately up to 90% of patients, whereas Reston EBOV has never caused lethal infection in humans (31) and is less pathogenic in experimentally infected nonhuman primates than Zaire EBOV (16). However, the factors that influence the different pathogenicity among filoviruses remain unclear.

The envelope glycoprotein (GP) of filoviruses is the only spike protein and is responsible for both receptor binding and membrane fusion. GP is comprised of two molecules, GP1 and GP2, which are linked by a disulfide bond. GP1 contains the receptor-binding domain, which is responsible for the viral attachment to cell surface molecules (9, 25). GP2 has the heptad repeat regions required for assembling GP as a trimer and the internal fusion loop, which is thought to interact with the cellular membrane (50). Although the trigger to promote the conformational change leading to membrane fusion is not fully understood, it was recently suggested that endosomal proteolysis of EBOV GP by cysteine proteases such as cathepsins B and L plays an important role in inducing membrane fusion (4).

Both MARV and EBOV GPs are heavily glycosylated and contain both N- and O-linked carbohydrate chains with different terminal sialylation patterns that seem to depend on the virus strains and cell lines used for virus propagation (12, 18,

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* Corresponding author. Mailing address: Department of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Kita-20, Nishi-10, Kita-ku, Sapporo 001-0020, Japan. Phone: 81-11-706-9502. Fax: 81-11-706-7310. E-mail: atakada@czc.hokudai.ac.jp.

† Present address: Laboratory of Virology, Rocky Mountain Laboratories, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT 59840.

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39, 48). The middle one-third of the GP molecule particularly varies among filoviruses and includes a mucin-like region (MLR) that contains a number of potential N- and O-linked glycosylation sites (32, 52). It is thought that carbohydrate chains on GP are recognized by cellular C-type lectins, such as the liver-specific C-type lectin asialoglycoprotein receptor (ASGP-R) (3), dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN), liver/lymph node-SIGN (L-SIGN) (1, 2, 20, 30, 33, 40), human macrophage galactose-type C-type lectin (hMGL) (46), and liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin) (8, 19, 20, 34). While these C-type lectins show different specificities, depending on the structures of target glycans, all have been reported to promote filovirus entry. Hepatocytes, dendritic cells (DCs), monocytes, and macrophages are thought to be the preferred target cells of filoviruses, and infection of these cells is important for hemorrhagic manifestation and immune disorders (7, 13, 15, 36). Thus, increased infection of these cells might be directly involved in the pathogenesis of filoviruses (16).

In the present study, using the vesicular stomatitis virus (VSV) pseudotype system (VSV that contains the green fluorescent protein [GFP] gene rather than the receptor-binding GP gene [VSVΔG*]) described previously (45), we compared the properties of Angola and Musoke GPs and found a significant difference in the ability to utilize hMGL and DC-SIGN for their entry. Importantly, GP binding affinity for the C-type lectins was not the primary factor contributing to the difference. We identified a single amino acid involved in the different efficiency of C-type lectin-mediated entry between Angola and Musoke. Three-dimensional analysis suggested that this amino acid might affect the processing of GP by endosomal cysteine proteases and/or flexibility of the GP internal fusion loop. Here, mechanisms underlying the different efficiencies for C-type lectin-mediated entry of filoviruses are discussed.

MATERIALS AND METHODS

Viruses and cells. VSVΔG* expressing GFP pseudotyped with MARV GPs was generated as previously described (45). The viruses were treated with neutralizing monoclonal antibody I1 to VSV G protein before use (28). The virus titer was determined by counting the number of cells expressing GFP using fluorescence microscopy or flow cytometry.

Vero E6 and HEK293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics. Human chronic myelogenous leukemia (K562) cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics. K562 clones expressing hMGL (K562/hMGL) were generated as previously described (46). cDNA encoding DC-SIGN was isolated from a placenta cDNA library (Invitrogen) and then cloned into a mammalian cell expression vector, pcDNA3.1(+) (Invitrogen). K562 cells were transfected with the plasmid using Attractene transfection reagent (Qiagen). After selection with Geneticin (G418 sulfate; Calbiochem), DC-SIGN-positive cells (K562/DC-SIGN) were enriched with immunomagnetic beads by using monoclonal antibody CD209 (Beckman Coulter). K562 cells transfected with the empty vector pcDNA3.1(+) and selected by Geneticin were used as control cells (K562/mock).

Expression of soluble recombinant hMGL and DC-SIGN. Soluble hMGL was purified by affinity chromatography on a column of galactose-Sepharose 4B as described previously (44). The expression plasmid pET-15b encoding the extracellular domain (ECD) of DC-SIGN was similarly constructed. The plasmid was subsequently used to transform *Escherichia coli* BL21/DE3 pLysS. The recombinant DC-SIGN ECD was prepared from inclusion bodies in *E. coli*. The recombinant DC-SIGN ECD was bound to mannose-Sepharose 4B and eluted with 10 mM EDTA. Subsequently, biotinylation of these soluble proteins was performed using EZ-Link sulfo-NHS-LC-biotin (Pierce).

Lectin-ELISA analysis. VSVΔG* pseudotyped with GPs was purified by ultracentrifugation through a 25% sucrose cushion and diluted to give a titer of 5×10^5 infectious units (IU)/ml in phosphate-buffered saline (PBS). Enzyme-linked immunosorbent assay (ELISA) plates were coated with the viruses and then blocked with 3% bovine serum albumin in PBS. After each well was washed with Dulbecco's Tris-buffered saline (dTBS), biotinylated hMGL or DC-SIGN in dTBS was added. To detect C-type lectins bound to the viruses, horseradish peroxidase (HRP)-streptavidin (Jackson ImmunoResearch) and 3,3′,5,5′-tetramethylbenzidine (Sigma) were used.

Mutagenesis. To construct the mutant GPs, MARV GP cDNAs were cloned into the pATX vector, kindly provided by H. Ebihara (Laboratory of Virology, Department of Health and Human Services, Rocky Mountain Laboratories, Division of Intramural Research, NIAID, NIH). By using the primers containing the sequences of the desired regions and the class IIS restriction enzyme, the BsmBI site, the MLR-deletion mutant, and chimeric GP constructs were generated. Mutant GPs with a single substitution (A/H504T, A/G547V, A/A596T, A/R618K, M/T504H, M/V547G, M/T596A, and M/K618R) were generated by using the primers containing the desired mutations and the BsmBI site. All the mutant GP genes were cloned into pCAGGS, the mammalian expression plasmid, and used for expression of the GPs in HEK293T cells.

Virus titration. The infectivity of VSVΔG* pseudotyped with GPs on K562 clones was determined by counting the number of GFP-positive cells using flow cytometry. To test C-type lectin-mediated entry, 10^5 cells of the K562 clones in 96-well plates were infected with the respective viruses, whose titers were standardized (i.e., all the viruses were diluted to give 1×10^5 to 5×10^5 IU/ml in Vero E6 cells that uniformly gave approximately 1×10^4 to 5×10^4 IU/ml in K562/mock cells), and the number of GFP-positive cells were counted. To investigate the effects of the cathepsin B and L inhibitors, CA-074Me and FY-dmk, respectively (Calbiochem), cells were treated with one of the inhibitors for 3 h before infection.

Binding assay. Approximately 10^6 infectious units (in Vero E6 cells) of purified VSVΔG* pseudotyped with GPs were incubated with 10^5 cells of K562/hMGL and K562/DC-SIGN for 1 h on ice. After being washed three times with PBS(+), cells were lysed to measure the amount of VSV matrix protein in the virions which bound on the cell surface.

SDS-PAGE and Western blotting. Cells or purified viruses were lysed with PBS containing 1% Triton X-100 and protease inhibitor cocktail Complete Mini (Roche), and the insoluble fraction was removed by centrifugation. Lysates were mixed with Laemmli sample buffer (Bio-Rad), electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 to 20% SuperSep (Wako), and blotted on a polyvinylidene difluoride (PVDF) membrane (Millipore). Non-specific binding to the membrane was blocked with 3% skim milk in PBS. A mixture of the sera obtained from mice immunized with Angola virus-like particles and Musoke virus-like particles for detecting MARV GPs or anti-VSV matrix protein monoclonal antibody (195-2) was incubated with the membrane. Peroxidase-conjugated AffiniPure goat anti-mouse IgG(H+L) (Jackson ImmunoResearch) and Immobilon Western (Millipore) were used for visualization. Intensities of specific bands were measured with ATTO CS Analyzer 2.1.

Molecular modeling. A three-dimensional model of Angola GP was generated by a homology modeling method using the crystal structure of EBOV GP (Protein Data Bank [PDB] code 3CSY) (27) as a template. The sequence alignment between MARV and EBOV GPs was based on that previously reported by Lee et al. (27). One hundred models of the first construction were generated using the automodel class in Modeller 9v6 (35), and the model with the lowest value of the Modeller objective function was selected. Next, to fill the gap of some potential loop conformation that the structural template of Zaire EBOV GP lacks (residues 174 to 197, 208 to 218, and 272 to 291 in the Angola GP numbering that correspond to residues 190 to 213, 224 to 225, and 279 to 298 in Zaire EBOV GP, respectively), two hundred models were generated by the loop model class (14). The best loop model was chosen by a combination of the Modeller objective function value and the discrete optimized protein energy (DOPE) statistical potential score (38). Then, the model, after addition of hydrogen atoms, was refined by energy minimization (EM) with the minimization protocols in the Discovery Studio 2.1 software package (Accelrys, San Diego, CA), using a CHARMM force field. Steepest descent, followed by conjugate gradient minimizations, was carried out until the root mean square (RMS) gradient was less than or equal to 0.01 kcal/mol/Å. The generalized Born implicit solvent model (43, 47) was used to model the effects of solvation. The model of Angola GP was finally evaluated by using PROCHECK (26), WHAT_CHECK (22), and Verify3D (10).

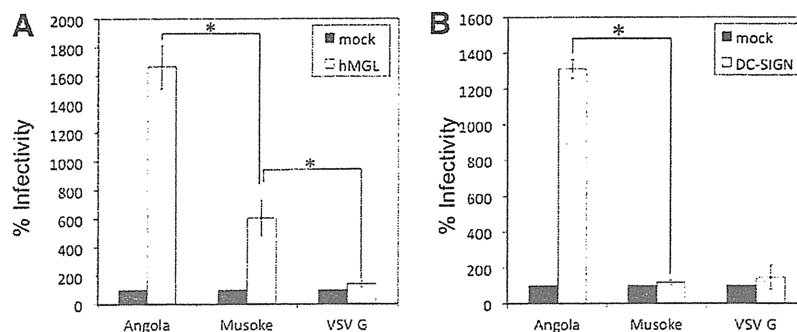

FIG. 1. Infectivity of VSVΔG* pseudotyped with MARV GPs in K562 cells expressing the C-type lectins. The infectivities of VSV-Angola and -Musoke were standardized using Vero E6 cells, as described in Materials and Methods, and approximately the same titers of viruses were used to infect K562/mock, K562/hMGL, and K562/DC-SIGN cells. The infected cells were counted using a flow cytometer, and the percentages of infectivity (i.e., relative infectivities) in K562/hMGL (A) and K562/DC-SIGN (B) cells were determined by setting the number of the infected K562/mock cells to 100% (46). All experiments were done in triplicate, and average results and standard deviations are shown. Statistical significance was determined by Student's *t* test. *, $P < 0.05$.

RESULTS

Efficiency of C-type lectin-mediated entry differs between MARV strains. We generated VSVΔG* bearing VSV G (VSV-VSV G), VSVΔG* bearing Angola GP (VSV-Angola), or VSVΔG* bearing Musoke GP (VSV-Musoke), and the infectivities of these viruses in K562/hMGL or K562/DC-SIGN cells were compared (Fig. 1). No significant enhancement of VSV-VSV G infectivity was seen in these C-type lectin-expressing cells. Consistent with a previous study (46), the viruses infected K562/hMGL cells more efficiently than they infected control K562/mock cells. In K562/DC-SIGN cells, the difference was observed only for VSV-Angola infectivity. It was noted that VSV-Angola showed significantly higher infectivity in these C-type lectin-expressing cells than VSV-Musoke, as was seen between Zaire and Reston EBOVs (46).

hMGL and DC-SIGN bind to MARV GPs in a different manner. To test the attachment of VSV-Angola and -Musoke to the surfaces of the cells expressing C-type lectins, a direct binding assay was performed (Fig. 2A). In both K562/hMGL and K562/DC-SIGN cells, only limited differences of the viruses attached on the cell surfaces were observed. For more quantitative analysis of the binding of MARV GPs to the C-type lectins, we next carried out a lectin-ELISA using soluble forms of hMGL and DC-SIGN and purified viruses (Fig. 2B and C). We found that both lectins bound to Angola and Musoke GPs in a dose-dependent manner and that hMGL had slightly higher ability to bind to Angola GPs than to bind to Musoke GPs, whereas DC-SIGN similarly bound to both GPs, confirming the different glycan specificities of these lectins (i.e., hMGL and DC-SIGN preferentially react with O-glycans and high-mannose-type N-glycans, respectively) (11, 21, 44).

MLRs and GP2 are important for efficient entry mediated by C-type lectins. MLRs of filoviruses have been shown to play an important role in interaction with the C-type lectins. To ascertain the contribution of the MLR of MARV GP to C-type lectin-mediated entry, we first constructed MLR-deletion mutants (AΔ and MΔ) (Fig. 3A) and examined the infectivities of VSVΔG* pseudotyped with these mutant GPs (VSV-AΔ and -MΔ) in Vero E6, K562/hMGL, and K562/DC-SIGN cells. VSV-AΔ and -MΔ showed no defects in their infectivities in Vero E6 cells, consistent with a previous study (32), indicating that glycosylation in the MLR and GP cleavage by furin are not essential to infect Vero E6 cells. In contrast, these viruses infected K562 cells expressing the C-type lectins much less efficiently than VSVΔG* pseudotyped with full-length wild-type GPs (Fig. 3B and C). These results indicate the major contribution of the MLR to C-type lectin-mediated entry of MARV.

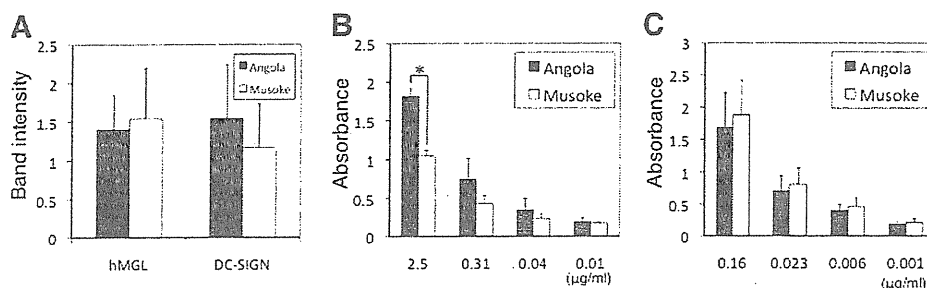

FIG. 2. Binding affinity of the C-type lectins to VSVΔG* pseudotyped with MARV GPs. (A) The amounts of VSV-Angola and -Musoke that attached on K562/hMGL or K562/DC-SIGN cells were shown as band intensities of VSV matrix protein. (B, C) A lectin-ELISA was performed, using purified VSV-Angola and -Musoke as antigens. Biotinylated recombinant soluble hMGL (B) and DC-SIGN ECD (C) were incubated at the indicated concentrations and visualized, as described in Materials and Methods. All experiments were done in triplicate, and average results and standard deviations are shown. Statistical significance was determined by Student's *t* test. *, $P < 0.05$.

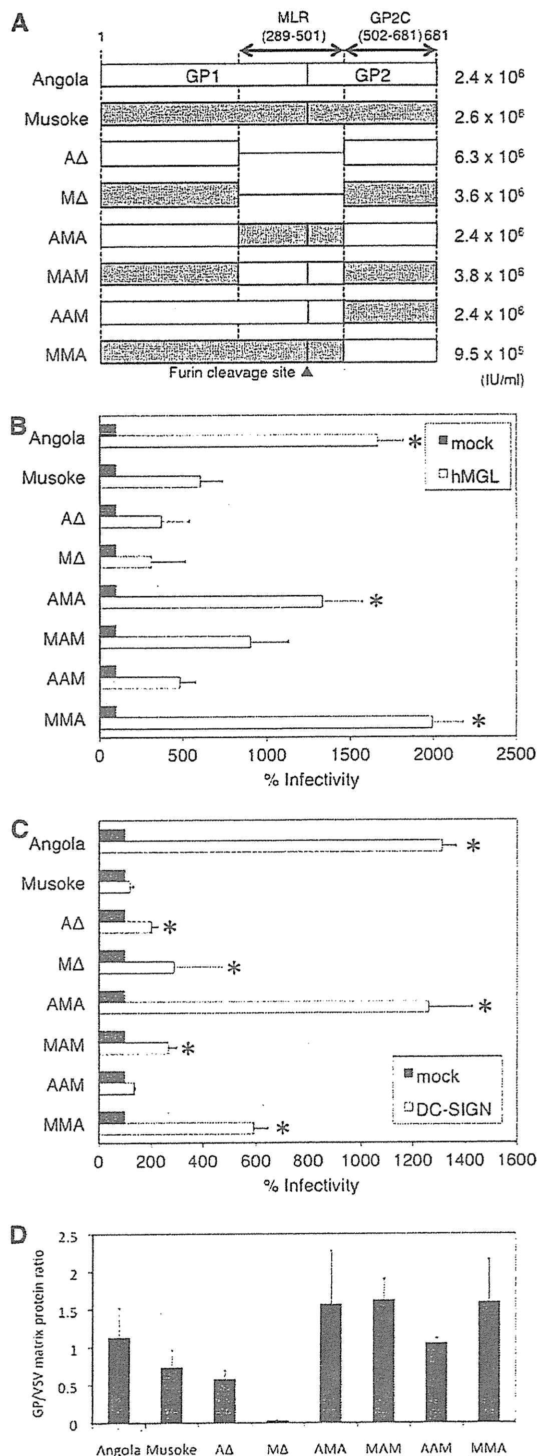

FIG. 3. Infectivity of VSVΔG* pseudotyped with the deletion or chimeric mutant GPs in C-type lectin-expressing cells. (A) The names of the MARV mutant GPs and the relevant amino acid positions are shown in the schematic. The number of infectious units determined for each virus in Vero E6 cells are shown on the right. (B, C) The relative infectivities of the viruses in K562/hMGL (B) and K562/DC-SIGN (C) cells were determined, as described in the legend of Fig. 1. All experiments were done in triplicate, and average results and standard deviations are shown. Statistical significance was determined (compared to the infectivity of VSV-Musoke in each K562/hMGL or K562/DC-SIGN cell) by Student's t test. *, $P < 0.05$. (D) The amounts of mutant GPs incorporated within the pseudotyped VSVΔG* were quantitated by using Western blotting of purified virions. Band intensities of MARV GPs and VSV matrix protein were determined, and their ratios are shown.

Next, we constructed chimeric GPs whose MLRs were swapped (AMA and MAM) (Fig. 3A), and the infectivities of VSVΔG* pseudotyped with these chimeric GPs (VSV-AMA and VSV-MAM) were tested (Fig. 3B and C). Unexpectedly, the relative infectivity of VSV-AMA in K562 cells expressing the C-type lectins was significantly higher than that of VSV-Musoke and similar to that of VSV-Angola. Replacement of the MLR of Musoke GP with that of Angola GP (MAM) showed only minimal effects on the enhancement of infectivity in the C-type lectin-expressing cells if compared with VSV-Musoke infectivity.

We finally replaced amino acid positions 502 to 681 of the GP2 regions (GP2C) of each (AAM and MMA) (Fig. 3A) and found that VSVΔG* pseudotyped with the chimeric Angola GP that had Musoke GP2C (VSV-AAM) infected both K562/hMGL and K562/DC-SIGN cells less efficiently than VSV-Angola (Fig. 3B and C), and the relative infectivities of VSV-AAM were similar to that of VSV-Musoke in both C-type lectin-expressing cell types. In contrast, the infectivities of VSV-MMA in both types of cells were significantly higher than that of VSV-Musoke or -AAM. These results indicate that GP2C is critical for the difference in the efficiency of C-type lectin-mediated entry between VSV-Angola and -Musoke. It was confirmed that all the chimeric GPs were similarly incorporated into virions (Fig. 3D). MΔ was not clearly detected by Western blotting. It was most likely due to the lack of the MLR containing many specific epitopes, resulting in the different reactivity of polyclonal serum to MΔ. We further confirmed that fully functional GPs were incorporated into VSV virions, since there is no significant difference in the infectivities in Vero E6 cells among these viruses.

Substitution of an amino acid at position 547 in the GP2 region influences the efficiency of C-type lectin-mediated entry. There are four different amino acids in GP2C between the Angola and Musoke GPs. To identify which amino acid(s) contributes to the different ability of C-type lectin-mediated entry between the Angola and Musoke strains, the following eight mutant GPs that contain single-amino-acid substitutions were constructed: four Angola-based mutant GPs (A/H504T, A/G547V, A/A596T, and A/R618K) and four Musoke-based mutant GPs (M/T504H, M/V547G, M/T596A, and M/K618R) (Fig. 4A). The infectivity levels of VSVΔG* pseudotyped with these mutant GPs in K562/hMGL or K562/DC-SIGN cells were compared (Fig. 4B and C). While the mutations at position 504, 596, or 618 did not affect the infectivity of the respective viruses in cells expressing the C-type lectins, the infectivities of VSVΔG* pseudotyped with mutant GPs with substitution at position 547 (VSV-A/G547V and -M/V547G) were clearly switched (i.e., the relative infectivities of VSV-Angola and -M/V547A in the C-type lectin-expressing cells were comparable and higher than those of VSV-Musoke and -A/G547V).

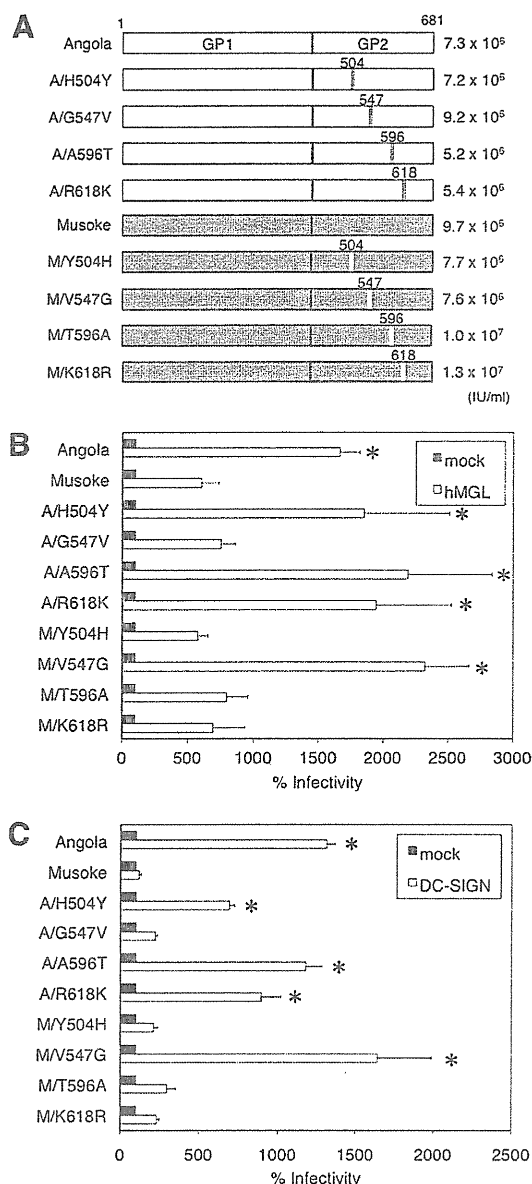

FIG. 4. Infectivity of VSVΔG* pseudotyped with the single-amino-acid mutant GPs in the C-type lectin-expressing cells. (A) The names of the MARV GP mutants and the positions of substituted amino acids are shown in the schematic. The number of infectious units determined for each virus in Vero E6 cells are shown on the right. (B, C) The relative infectivities of the viruses in K562/hMGL (B) and K562/DC-SIGN (C) cells were determined, as described in the legend of Fig. 1. All experiments were done in triplicate, and average results and standard deviations are shown. Statistical significance was determined (compared to the infectivity of VSV-Musoke in each K562/hMGL or K562/DC-SIGN cell) by Student's t test. *, $P < 0.05$.

Infectivities of VSV-Angola and -Musoke are reduced in different manners by cathepsin inhibitors in the C-type lectin-expressing cells. Because the GP binding affinity for C-type lectins (i.e., attachment) did not seem essential for the different ability levels of C-type lectin-mediated entry between the Angola and Musoke strains, we then focused on the following steps of viral entry. In EBOV and human coronavirus entry, it has been reported that proteolysis of GP by cellular endosomal cysteine proteases cathepsins B and/or L is necessary (4, 23, 24, 37). A three-dimensional model of Angola GP revealed that glycine at position 547 of Angola GP is presumed to form a β strand included in the internal fusion loop and is in close proximity to the putative cathepsin processing site (Fig. 5) (9, 27). Thus, we hypothesized that the amino acid change at position 547 affected the efficiency of cathepsin processing, which might lead to membrane fusion.

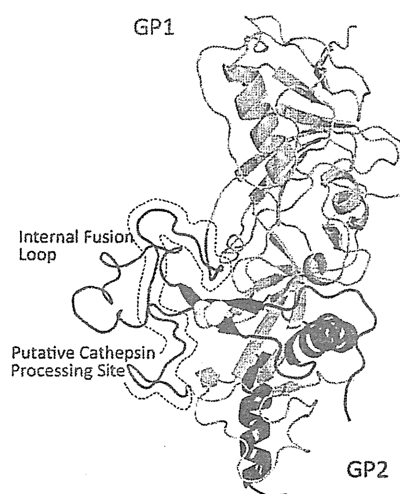

FIG. 5. Three-dimensional structure of the Angola GP monomer. The crystal structure of EBOV GP (PDB code 3CSY) was used as a template for homology modeling. GP1 (lime green) and GP2 (dark blue) are shown as ribbon models. Glycine at position 547 (G547, yellow) is shown as a space-filling model. The putative cathepsin cleavage site (amino acid residues 174 to 197 of Angola GP, corresponding to amino acid residues 190 to 213 of Zaire EBOV GP) is colored in red. This figure was prepared using PyMOL (DeLano Scientific LLC).

To test our hypothesis, VSVΔG* pseudotyped with wild-type or mutant (A/G547V and M/V547G) GPs was analyzed by comparing the infectivities of these viruses in Vero E6, control K562/mock, K562/hMGL, and K562/DC-SIGN cells pretreated with cathepsin inhibitors (Fig. 6). The infectivities of all the viruses were reduced by both of the inhibitors in a dose-dependent manner in all cells tested, suggesting that the proteolysis of GP by cathepsins B and/or L is also required for MARV entry. Interestingly, the infectivities of VSV-Angola and -M/V547G in K562 cells expressing the C-type lectins were less effectively reduced by these cathepsin inhibitors than those of VSV-Musoke and -A/G547V, whereas in Vero E6 and K562/mock cells, the differences in the infectivity at each concentration of the inhibitors were limited among the viruses. Paired Student's t test revealed significant differences ($P < 0.01$) in the following pairs: VSV-Angola and -Musoke, VSV-Angola and -A/G547C, and VSV-Musoke and -M/V547G (Fig. 6C, G, and H). And in Fig. 6D, the significant differences among these viruses were observed at a concentration of 10 μM by Student's t test. The infectivities of VSVΔG* pseudotyped with the other mutant GPs (i.e., VSV-A/H504T, -A/A596T, -A/

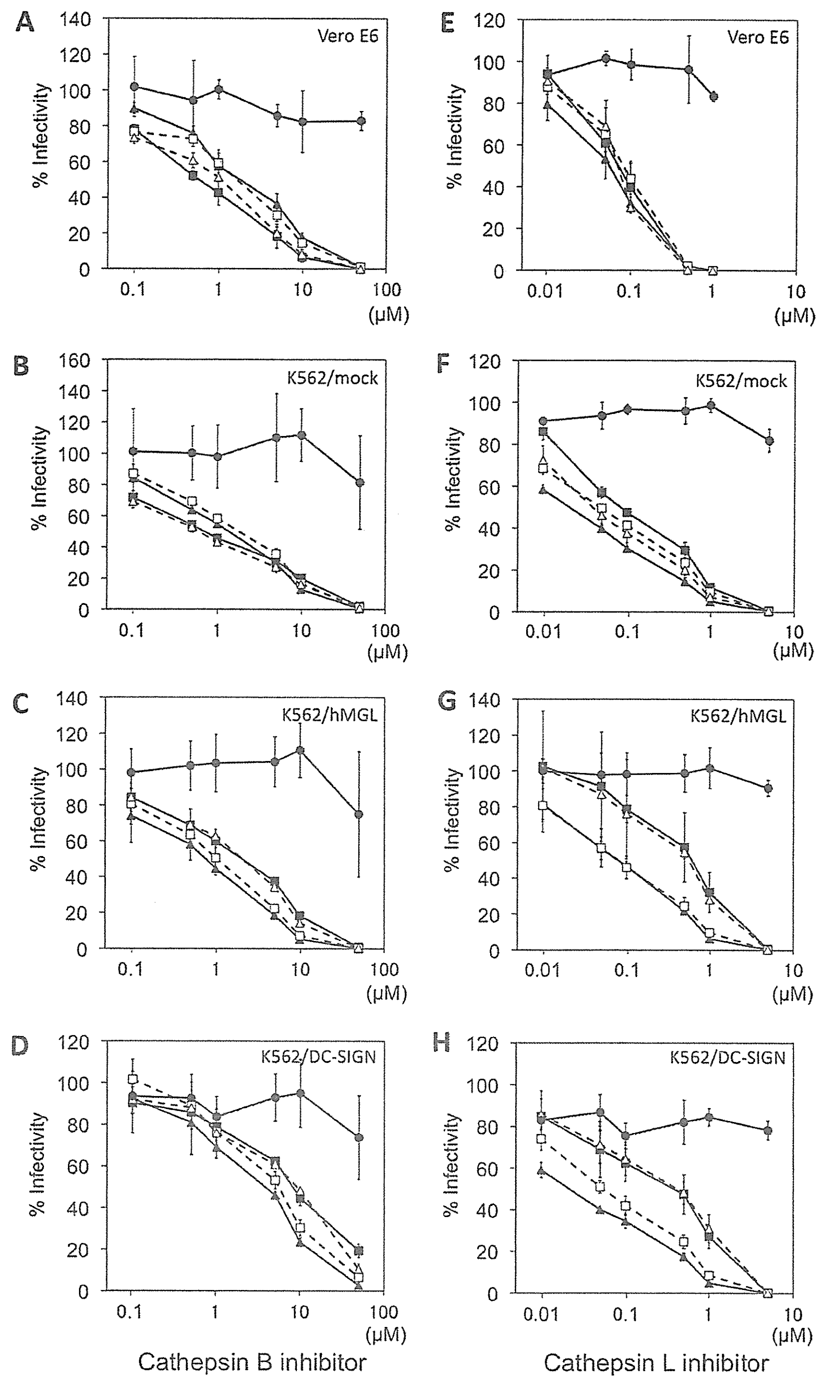

FIG. 6. Inhibition of virus infectivity by cathepsin inhibitors. Vero E6 (A and E), K562/mock (B and F), K562/hMGL (C and G), and K562/DC-SIGN (D and H) cells were pretreated with cathepsin B inhibitor (A, B, C, and D), cathepsin L inhibitor (E, F, G, and H), or dimethyl sulfoxide (DMSO) for 3 h and then infected with VSV-Angola (closed square), -Musoke (closed triangle), -A/G547V (open square), -M/V547G (open triangle) or -VSV G (closed circle). The number of infected cells, given in DMSO-treated cells, was used to set the 100% infectivity level for each cell type, and the relative infectivity was determined at each concentration of the inhibitors. All experiments were done in triplicate, and average results and standard deviations are shown.

R618K, -M/T504H, -M/T596A, and -M/K618R) were reduced by these cathepsin inhibitors similar to those of VSVΔG* pseudotyped with the respective wild-type GPs (data not shown).

DISCUSSION

It has been shown that cellular C-type lectins are utilized for the attachment of several viruses to host cells. hMGL, which

was originally founded as a macrophage-specific C-type lectin recognizing galactose/*N*-acetylgalactosamine, is expressed in monocyte-derived DCs and macrophages (44), and it was demonstrated that hMGL promoted the infection of EBOV and MARV (46). DC-SIGN, which recognizes high-mannose-type N-glycans and plays an important role in regulating the immune system (53), has also been shown to promote infection by several viruses (e.g., Ebola, Marburg, human immunodeficiency, hepatitis C, measles, dengue, and influenza viruses) (49, 53). A liver-specific C-type lectin, ASGP-R, which recognizes galactose in carbohydrate side chains, has been shown to be exploited for MARV infection of hepatocytes (3) and is thought to be one of the possible determinants of hepatotropism of MARV (17). Taken together, increased infection of these cells expressing C-type lectins and subsequent destruction of the host immune functions and coagulation system may be crucial for the pathogenesis of filoviruses. Our previous study (46) and the present study show that the different abilities used to utilize the C-type lectins among filoviruses to promote cellular entry might be correlated with the pathogenicities of the viruses. *In vivo* study may be needed to provide a direct link between the pathogenicity of MARV and its ability to use C-type lectins for entry into target cells.

Using deletion mutant GPs, we found that both hMGL and DC-SIGN principally recognized MLRs. Amino acid comparison between Angola and Musoke GPs indicates that the similarity of their MLRs is 86.4% (data not shown). The numbers of potential O-glycosylation sites vary between Angola and Musoke GPs (the number of these sites for Musoke GP is less than the number of these sites for Angola GP), whereas the potential N-glycosylation sites are relatively conserved, supporting our observation that hMGL, but not DC-SIGN, bound more efficiently to Angola GP than to Musoke GP in the lectin-ELISA. However, using chimeric mutant GPs, we showed that the structure of the MLR itself was not essential for the different levels of infectivity between VSV-Angola and -Musoke in K562 cells expressing these C-type lectins, suggesting that the capacity of the GP for binding to C-type lectins through the MLR (i.e., the glycosylation pattern of MLR) is not the only factor contributing to the efficiency of C-type lectin-mediated entry. Indeed, we identified that the amino acid at position 547 in GP2, but not in the MLR, was critical for the different efficiency levels of C-type lectin-mediated entry between VSV-Angola and -Musoke. It is of interest to confirm the importance of this amino acid in MARV infection by using a reverse genetics approach.

In our three-dimensional model, it seemed unlikely that glycine/valine at position 547 directly influenced the binding to the putative specific receptors, since substitution of this amino acid did not affect the infectivity of pseudotyped viruses in Vero E6 or control K562 cells (data not shown), and this amino acid was not located around the putative receptor-binding domain (9, 25). We showed that the effects of cathepsin inhibitors on the infectivity differ between Angola and Musoke only in the C-type lectin-expressing cells, and the single-amino-acid substitution at position 547 altered the effects of cathepsin inhibitors. However, no significant difference in the susceptibility to cathepsins was seen in direct digestion assays *in vitro* using soluble forms of the C-type lectins and purified virions (data not shown). These results suggest that glycine at position 547 increases sensitivity of GPs to endosomal cathepsins during C-type lectin-mediated entry, but our *in vitro* digestion assay did not provide actual conditions for interaction between GPs and C-type lectins in endosomes. It may be also possible that when the viruses are internalized to endosomes through the interaction with C-type lectins, (i) the amino acid at position 547 affects the flexibility of the fusion loop and/or the efficiency of conformational change, and (ii) glycine at position 547 weakens GP1-GP2 interaction, resulting in reduced cathepsin dependence in virus entry, as reported with EBOV GP (51). It is interesting to clarify how this amino acid contributes to the intramolecule interaction required for GP functions.

In summary, our data suggest that the efficiency of C-type lectin-mediated entry of filoviruses is determined not only by direct interaction between GP and C-type lectins but also by some mechanisms underlying endosomal entry, such as proteolytic processing, and likely by cathepsins or membrane fusion machinery. Although further investigations are required to prove our hypotheses, this study provides new insights into understanding the molecular basis of the C-type lectin-mediated entry of filoviruses, which may have a possible link to their pathogenicity.

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学内グラント 終了時報告書

平成20-21年度　学内グラント報告書

ペプチド結合リポソームを用いた，エボラウイルスに対するCTL誘導型ワクチンの開発

研究代表者　松井　政則（埼玉医科大学　医学部　微生物学）
研究分担者　禾　泰壽[1]，山岸　敏之[2]，赤塚　俊隆[3]，内田　哲也[4]

緒言

エボラウイルスは，フィロウイルス科に属するRNAウイルスで，ヒトを含む霊長類に重篤な新興感染症であるエボラ出血熱を引き起こす．その致死率は時に90%を超え，最も危険なウイルスである[1]．1976年にスーダンとザイールで大流行して以来，中央・西アフリカで散発的に流行を繰り返しているが，米国においても1989年にサル検疫施設で発生し大問題となった．致死量が極めて少量で，血液や体液を介してヒトからヒトへ感染するため極めて脅威であり，Biosafety level 4 (BSL4) の封じ込めが必要である．自然宿主はコウモリの可能性が示唆されている[2]が，まだ特定されていない．我が国ではまだ発症例はないが，ペットのサルの輸入や旅行者の増加に伴い，日本に侵入する危険性は高まっており，一類感染症に指定されている．また，米国政府は，エボラウイルスをバイオテロに利用しうるカテゴリーAの生物兵器と位置づけている．しかしながら，効果的な予防・治療法は存在しない．以上の状況から，地球規模で，エボラウイルスに対するワクチンの開発は急務である．

エボラ出血熱の患者では，激しいリンパ球のアポトーシスがみられ，免疫機構がうまく働かない．しかし，生き延びたわずかの感染者では，ウイルス特異的細胞傷害性T細胞（CTL）やウイルスに対する抗体が存在する[3]．従って，エボラウイルス感染においても，一般的なウイルス感染症と同様に，体液性免疫及び細胞性免疫が有効にウイルスを排除すると考えられる．事実，マウスにウイルス中和抗体[4]やウイルス特異的CTL[5]を導入することによって，エボラウイルスへの抵抗性が示された．一方，サルを用いた実験では，細胞性免疫は有効にウイルスを排除した[6-8]が，中和抗体はウイルスをコントロールできなかった[9]．従って，CTLを中心とした細胞性免疫がエボラウイルスの排除に重要である可能性が高く，CTL誘導型ワクチンの開発が必要であると思われる．しかし，エボラ出血熱における細胞性免疫の研究はほとんど行われておらず，そのCTLエピトープはわずか3つしかわかっていない[10]．

エボラウイルスを扱うにはBSL4の封じ込めが必要であり，それが研究上の大きな障害である．我々は，組換えウイルスとMHCクラスIトランスジェニックマウスを用いることで，BSL3やBSL4実験施設が必要とされる危険なウイルスに対しても，BSL2実験室でCTLのエピトープを同定できる方法を開発した．さらに，エピトープに相当する短いペプチドをリポソーム表面に結合させてマウスに免疫することで，極めて強力にペプチド特異的CTLを誘導できることを示した[11]．我々は，これらの方法を使って，BSL3の封じ込めが必要な，重症急性呼吸器症候群（SARS）コロナウイルス[12,13]，及びH5N1亜型高病原性鳥インフルエンザウイルス[14]のHLA-A2拘束性CTLエピトープを多数同定した．そして，同定したエピトープを結合したペプチド結合リポソームを作製し，HLA-A2トランスジェニックマウスに免疫して，ウイルス特異的CTLの誘導を詳細に検討した[12-14]．現在，我々は，これらのデータを基にして，新型インフルエンザに対する新しいワクチンの開発・実用化をめざした研究を続けている（特許2件出願中）．

本研究では，同様の方法を用いて，エボラウイルスに対するCTLエピトープを多数同定し，そのエピトープを利用してペプチド結合リポソームを作製・解析して，エボラ出血熱に対するワクチン開発のための基礎

1) 埼玉医科大学　医学部　分子生物学
2) 埼玉医科大学　医学部　解剖学
3) 埼玉医科大学　医学部　微生物学
4) 国立感染症研究所

データを得ることを目的とした．

材料と方法

1) HLA-A 2トランスジェニックマウス

マウスMHCクラスIとβ2-マイクログロブリン（β2-m）をノックアウトしたマウスに，ヒトMHCクラスIの一つであるHLA-A*0201（HLA-A2）とヒトβ2-m遺伝子を導入したトランスジェニックマウス（HHDマウス）[15]を使用した．HHDマウスは，仏・パスツール研究所・Lemonnier博士より供与された．

2) コンピューターによるCTLエピトープの予測

エボラウイルス（ザイール型）を構成する7つのタンパク質（NP, VP35, VP40, GP, VP30, VP24, L）のアミノ酸配列において，HLA-A2結合ペプチドモチーフに従い，2種類のコンピュータープログラム，「BIMAS」と「SYFPEITHI」で，9個のアミノ酸からなる94種類のCTLエピトープを予測した．これらのエピトープに相当するペプチドは，オペロン社により人工合成された．

3) HLA-A 2分子への結合アフィニティの測定

それぞれのペプチドのHLA-A2分子に対する結合アフィニティを，HLA-A2遺伝子を導入・発現させたTAP欠損細胞株，RMA-S-HHDを使ったpeptide binding assayで測定した．RMA-S-HHD細胞にさまざまな濃度のペプチドを加え，どの程度安定したHLA-A2分子が検出できるかを，抗HLA-A2モノクローナル抗体で染色しフローサイトメトリーで測定して，結合アフィニティを測定した．

4) 合成ペプチドによるIFN-γ^+CD 8$^+$ T細胞の誘導

ナイーブなHHDマウスの脾細胞を調整し，予測したエピトープに相当する合成ペプチドでパルスした．その細胞を，別のHHDマウスの尾静脈に細胞移入して免疫した．免疫7日後に，脾細胞を調整し，各々のペプチドで抗原刺激した．その後，細胞表面をFITC-抗CD8抗体，細胞内部をPE-抗IFN-γ抗体で染め，それぞれのエピトープに特異的に反応するIFN-γ^+CD8$^+$T細胞数を，フローサイトメトリーで測定した．

5) ペプチド結合リポソームによるCTLの誘導

IFN-γ^+CD8$^+$ T細胞を誘導したペプチドを，リポソーム表面に結合させてペプチド結合リポソームを作製し，CpGと混ぜてHHDマウスの皮下に免疫した．そして，免疫7日後に，以下の方法により，インフルエンザウイルス特異的CTLが誘導できるかどうかを検討した．

a) 細胞内サイトカイン陽性CTLの測定

免疫7日後に，マウス脾細胞を各々のペプチドで抗原刺激した．その後，細胞表面をFITC-抗CD8抗体，細胞内部をPE-抗IFN-γ 抗体で染め，それぞれのエピトープに特異的に反応するIFN-γ^+CD8$^+$ T細胞数を，フローサイトメトリーで測定した．

b) in vivo CTL assay

ナイーブなマウスの脾臓から，ペプチドでパルスした脾細胞とパルスしていない脾細胞を調整して，それぞれを異なる濃度のCFSEで染色した．その2つの細胞群を同数混ぜて，ペプチド結合リポソームで免疫したマウスに尾静脈から細胞移入し，ペプチドでパルスした脾細胞が特異的にどのくらい溶解されるかをフローサイトメトリーで測定した．

（倫理面への配慮）

マウスは，埼玉医大・実験動物管理運営規定に基づき飼育され，苦痛の軽減，安楽死等に配慮した指針に従って実験を行った．

結果

1) CTLエピトープの予測

エボラウイルス（ザイール株）を構成する7つのタンパク質（NP, VP35, VP40, GP, VP30, VP24, L）のアミノ酸配列から，HLA-A2拘束性エピトープの可能性が高い，9個のアミノ酸からなるペプチド配列を，2種類のエピトープ予測プログラム（BIMAS & SYFPEITHI）を用いて予測した．NPから13種類，VP35から10種類，VP40から4種類，GPから10種類，VP30から3種類，VP24から9種類，Lから45種類（計94種類）予測し，その合成ペプチドを作製した．

2) HLA-A 2分子への結合アフィニティの測定

予測したペプチドのHLA-A2分子への結合アフィニティを，RMA-S-HHD細胞を使って測定したところ，ほとんどのペプチドで高い結合アフィニティを示し，予測が良好であることがわかった．その中でも，極めて結合アフィニティが高い（BL50<100 μM）ペプチドが25種類存在した．

3) 予測したエピトープによるIFN-γ^+CD 8$^+$T細胞の誘導

ナイーブなHHDマウスから脾細胞を取り出して，94種類の予測エピトープに相当するペプチドでパルスし，別のHHDマウスの静脈に移入して免疫した．免疫したマウスの脾細胞を調整して，各々のペプチドでin vitroで抗原刺激し，フローサイトメトリーで細胞内IFN-γ^+CD8$^+$T細胞の誘導を測定した．その結果，94種類のうち24種類のペプチド（NP, 3種類；VP35, 1種類；VP40, 0種類；GP, 4種類；VP30, 3種類；VP24, 1種類；L，12種類）が，ペプチド特異的にIFN-γ^+CD8$^+$T細胞を誘導した．これらの細胞は，ペプチド特異的に活性化されたCTLと相同である考えられるため，これらのペプチドは，エボラウイルス由来のCTLエピトープであると推定される．

4) ペプチド結合リポソームによるIFN-γ^+CD 8$^+$ T細胞の誘導

24種類のエボラウイルス由来ペプチドが，特異

的にCTLを活性化したため，これらのペプチドをリポソームに結合させて，24種類のペプチド結合リポソームを作製した．そして，それらをCpGと共に，HHDマウスの皮下（footpad）に免疫した．その後，ペプチド特異的に誘導されるIFN-γ $^{+}$CD8^{+} T細胞の数を測定した．その結果，14種類のペプチド結合リポソーム（Lip-NP-6, -NP-7, -VP40-2, -GP-3, -GP-4, -GP-7, -GP-10, -VP30-2, -L-10, -L-12, -L-19, -L-22, -L-29, -L-39）で，有意にペプチド特異的IFN-γ $^{+}$CD8^{+} T細胞が誘導された（Fig. 1）．特に，NP-6, VP30-2, L-10, L-12, L-19, L-29を結合させたリポソームは強い誘導能を示した（Fig. 1）．

5）ペプチド結合リポソームによるCTL killing活性の誘導

次に，IFN-γ $^{+}$CD8^{+}T細胞を強く誘導した6種類のペプチド結合リポソーム（Lip-NP-6, -VP30-2, -L-10, -L-12, -L-19 , -L-29）について，マウス*in vivo*におけるCTL killing活性を調べた．その結果，やはり，6つのペプチド結合リポソームで極めて良好にペプチド特異的なkilling活性がみられた（Fig. 2）．また，IFN-γ $^{+}$CD8^{+}T細胞を特に強く誘導した3つのペプチド結合リポソーム（Lip-L-12, -L-19, -L-29）は，killing活性の誘導も強いことが示された（Fig. 2）．

考察

エボラウイルスを構成するタンパク質から，24種類のCTLエピトープが同定された．これらのエピトープとリポソームの結合物は，効率よくペプチド特異的にIFN-γ $^{+}$CD8^{+}T細胞を誘導し強いkilling活性を示

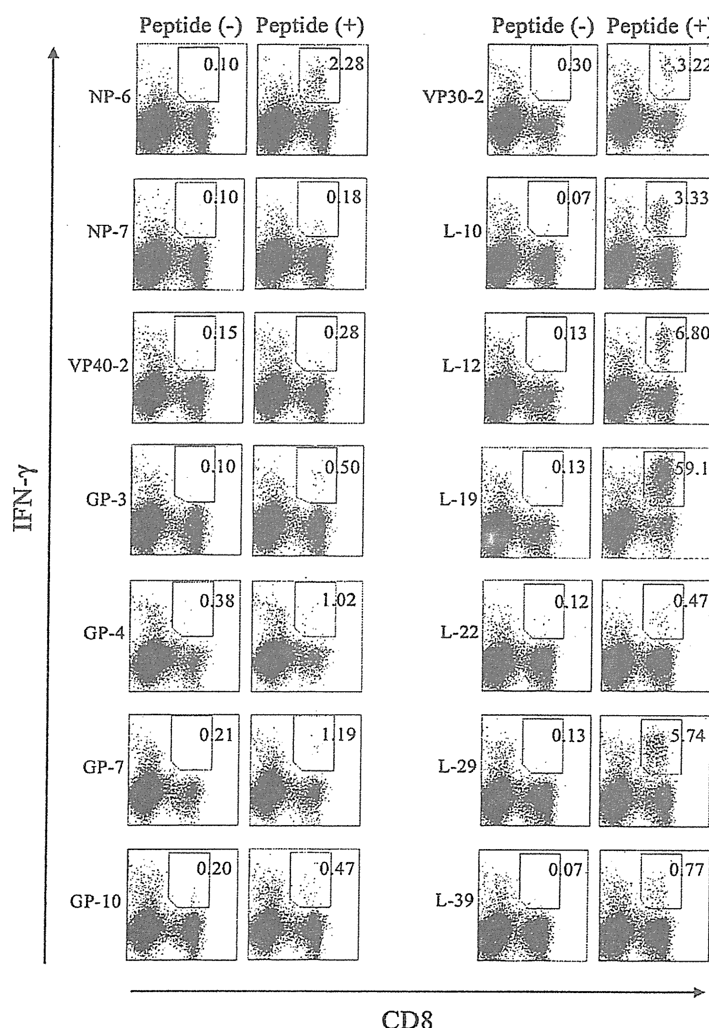

Fig. 1. Intracellular IFN-γ staining of CD8^{+} T cells specific for peptides derived from Ebola virus in mice immunized with surface-linked liposomal peptides. HHD mice were immunized once with either Lip-NP-6, -NP-7, -VP40-2, -GP-3, -GP-4, -GP-7, -GP-10, -VP30-2, -L-10, -L-12, -L-19, -L-22, -L-29, or -L-39 together with CpG.. After one week, spleen cells were prepared and stimulated with (+) or without (−) a relevant peptide for 5 h. Cells were then stained for their surface expression of CD8 (x axis) and their intracellular expression of IFN-γ (y axis). The numbers shown indicate the percentages of intracellular IFN-γ $^{+}$ cells within CD8^{+}T cells.

した．特に3つのペプチド結合リポソーム，Lip-L-12, -L-19，-L-29は，極めて強い活性を示した．従って，これらは，エボラウイルスに対して，効果の高いワクチンになりうる可能性が示唆された．エボラウイルスには，病原性の異なる4種（ザイール，スーダン，アイボリーコーストおよびレストン）が存在する．本研究では，もっとも致死率の高いザイールエボラウイルスのアミノ酸配列をもとに，CTLエピトープを同定したが，L-29は，4種類のエボラウイルスに共通のエピトープであった．従って，Lip-L-29は，どのエボラウイルスに対しても，防御効果を示すことが予想される．本研究では，実際にエボラウイルスを使ったウイルス実験を行わなかった．しかし，現在，北海道大学・人獣共通感染症リサーチセンターとの共同研究を開始しており，その共同研究の一環として，今後，カナダのBSL4施設でエボラウイルスを扱ったウイルスチャレンジ実験を行い，ペプチド結合リポソームのワクチン効果を検討する予定である．

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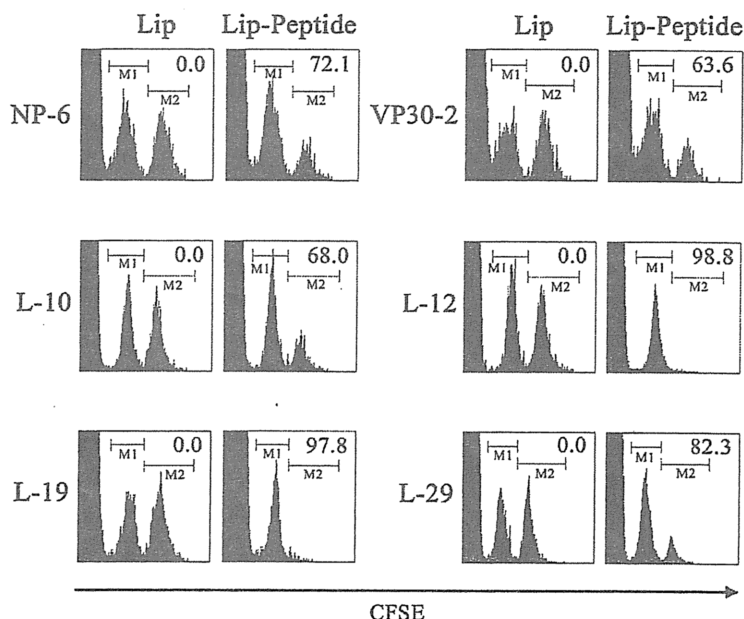

Fig. 2. CTL killing activities specific for peptides derived from Ebola virus in mice immunized with surface-linked liposomal peptides. HHD mice were immunized once with either each liposomal peptide (Lip-peptide) or liposomes alone (Lip) together with CpG. One week later, an equal number of a relevant peptide (NP-6, VP30-2, L-10, L-12, L-19 or L-29)-pulsed CFSE^{high} targets (M2) and unpulsed CFSE^{low} targets (M1) were transferred into the immunized mice by i.v. injection. After 12 h, CFSE-labeled cells were recovered from spleens of recipient mice and analyzed by flow cytometry. The numbers indicate the percentages of specific lysis.

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