Arch Virol (2016) 161:1447–1454
DOI 10.1007/s00705-016-2803-1

ORIGINAL ARTICLE

Analysis of the entry mechanism of Crimean-Congo hemorrhagic fever virus, using a vesicular stomatitis virus pseudotyping system

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Received: 24 September 2015 / Accepted: 21 February 2016 / Published online: 3 March 2016

Abstract Crimean-Congo hemorrhagic fever (CCHF) is a tick-borne disease causing severe hemorrhagic symptoms with a nearly 30 % case-fatality rate in humans. The experimental use of CCHF virus (CCHFV), which causes CCHF, requires high-biosafety-level (BSL) containment. In contrast, pseudotyping of various viral glycoproteins (GPs) onto vesicular stomatitis virus (VSV) can be used in facilities with lower BSL containment, and this has facilitated studies on the viral entry mechanism and the measurement of neutralizing activity, especially for highly pathogenic viruses. In the present study, we generated high titers of pseudotyped VSV bearing the CCHFV envelope GP and analyzed the mechanisms involved in CCHFV infection. A partial deletion of the CCHFV GP cytoplasmic domain increased the titer of the pseudotyped VSV, the entry mechanism of which was dependent on the CCHFV envelope GP. Using the pseudotype virus, DC-SIGN (a calcium-dependent [C-type] lectin cell-surface molecule) was revealed to enhance viral infection and act as an entry factor for CCHFV.

Introduction

Crimean-Congo hemorrhagic fever (CCHF) is a potentially fatal tick-borne infectious disease that has been reported in over 30 countries in parts of Africa, Eastern Europe, and Asia [1–5]. Human infection can occur through the bite of an infected tick or through contact with the tissue or blood of viremic animals or CCHF patients. The case-fatality rate is nearly 30 % [1–4, 6]. Both the incidence and geographic range of confirmed CCHF cases have increased [1, 5]. In Turkey, the first cases of CCHF were identified in 2002, after which the number of patients increased, and the number of identified cases is currently more than 9,000 [4]. In Iran, 1,017 cases occurred between 2000 and 2014 [4]. In China, 286 cases were identified between 1965 and 1997 [7]. In Kazakhstan, during 2000 to 2013, 212 cases were reported [8]. At present, there are no established countermeasures to combat CCHF.

CCHF is caused by infection with CCHF virus (CCHFV), which is a member of the genus *Nairovirus* in the family *Bunyaviridae* [9]. In most countries, experimental use of CCHFV requires biosafety level (BSL) 4 containment. The virus has a tri-segmented, negative-sense, single-stranded RNA genome and forms enveloped virions. The L, M, and S segments encode the RNA-dependent RNA polymerase, the envelope glycoprotein (GP), and the nucleocapsid protein, respectively. The precursor of GP is cleaved and modified to generate the structural proteins Gn and Gc and the non-structural proteins GP38 and NSm [10–12]. As the structural proteins form complexes on the envelope and interact with host receptors to initiate infection, they are the primary targets of neutralizing antibodies [13]. It was reported previously that the early stage of CCHFV infection is dependent on pH and cholesterol [14, 15]. Furthermore, it was previously shown that the Gc cytoplasmic tail of some viruses of the family *Bunyaviridae*, such as Rift Valley fever virus, Uukuniemi virus and Bunyamwera virus, is crucial for the trafficking and localization of not only Gc but also Gn [16–18].

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Vesicular stomatitis Indiana virus (VSV), a member of the family *Rhabdoviridae,* is a non-segmented, negative-sense, single-stranded RNA virus that can be handled in facilities with BSL-2 containment. Pseudotyping of viral glycoproteins onto VSV has facilitated studies of viral entry and measurement of virus neutralizing activity under BSL-2 containment [19–26]. As there are fewer than 50 BSL-4 containment facilities in the world, these pseudotype viruses are valuable tools, especially for the study and diagnosis of highly pathogenic viruses.

Although nucleolin has been reported to be a candidate receptor for CCHFV [27], it is still unclear which factors are required for CCHFV entry factors. Calcium-dependent (C-type) lectins recognize glycans, which exist as GPs and/or glycolipids, and some lectins such as DC-SIGN, LSECtin, MGL, and CLEC5A are known to be entry factors for several viruses, including human immunodeficiency virus 1 [28], measles virus [29], dengue virus [30], severe acute respiratory syndrome coronavirus (SARS-CoV) [31], filoviruses [31], and Lassa virus [32]. It has been shown that some members of the family *Bunyaviridae*, including the phleboviruses Rift Valley fever virus, Uukuniemi virus [33], and severe fever with thrombocytopenia syndrome virus (SFTSV) [34] use DC-SIGN as a receptor, and Uukuniemi virus was shown to bind directly to DC-SIGN [33]. Recognition of glycans on virions by C-type lectins results in the enhancement of viral endocytosis, after which the cellular and viral membranes fuse. CCHFV Gn and Gc have some N-glycosylated sites [10, 35] and are likely to bind to C-type lectins.

In the present study, in facilities with BSL-2 containment, we generated high titers of pseudotyped VSV bearing CCHFV envelope GP on its surface (CCHFVpv) and analyzed the mechanisms of CCHFV cell entry using the pseudotype virus.

Materials and methods

Cells

293T cells (ATCC CRL-3216), Vero cells (ATCC CCL81), and VeroE6 cells (ATCC CRL-1586) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10 % (v/v) fetal bovine serum (FBS) (Gibco). Jurkat cells (ATCC TIB-152) were cultured in RPMI-1640 (Sigma) medium supplemented with 10 % (v/v) FBS. Jurkat cells stably expressing fCD2ΔCT, DC-SIGN, and LSECtin were prepared as described previously [32].

Plasmids

cDNAs encoding the open reading frame of CCHFV IbAr 10200 strain GP (NP_950235) were cloned into pCAGGS using a Rapid DNA Ligation Kit (Roche) to generate pC-CCHFV GP. For constructing plasmids encoding mutant GPs in which the carboxyl terminal region was partially truncated, stop codons were introduced in pC-CCHFV GP by PCR-based mutagenesis to express the mutant GPs listed in Table 1. All plasmid constructions were confirmed by sequencing, using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Production of pseudotyped VSV bearing CCHFV envelope GP

Pseudotyped VSV bearing CCHFV envelope GP was generated as described previously [25, 26]. Briefly, 293T cells transfected with CCHFV GP plasmids using *Trans*IT-LT1 (Mirus) were infected with VSVΔG/GFP-*G or VSVΔG/Luc-*G, in which the G gene was replaced with the green fluorescent protein (GFP) or the luciferase gene, respectively, at a multiplicity of infection of 0.1-1. The virus was adsorbed for 2 h at 37 °C and then removed by extensively washing four times with serum-free DMEM. After 24 h of incubation at 37 °C with the culture medium, the culture supernatants were centrifuged to remove cell debris and stored until use at -80 °C. To produce a pseudotype virus (with the GFP gene or the luciferase gene) bearing no viral envelope proteins, empty plasmid pCAGGS or GFP expression plasmid pC-GFP was used. VSVpv/Luc (pseudotyped VSV with the luciferase gene bearing VSV G), MLVpv/Luc (pseudotyped VSV with the luciferase gene bearing murine leukemia virus envelope proteins), Lassapv/GFP (pseudotyped VSV with the GFP gene bearing Lassa virus envelope protein), and EBOVpv/GFP (pseudotyped VSV with the GFP gene bearing Ebola

Table 1 CCHFV GP variants and titers of pseudotyped VSV

Variant	Amino acid sequence of the cytoplasmic region	Titer (IU/ml)
Full-length	CFKCCRRTRGLFKYRHLKDDEETGYRRIIEKLNNKKGKNKLLDGERLADRRIAELFSTKTHIG	200
del4	CFKCCRRTRGLFKYRHLKDDEETGYRRIIEKLNNKKGKNKLLDG	1,150
del9	CFKCCRRTRGLFKYRHLKDDEETG	1,700
del10	CFKCCRRTRG	6,250
del14	C	1,225

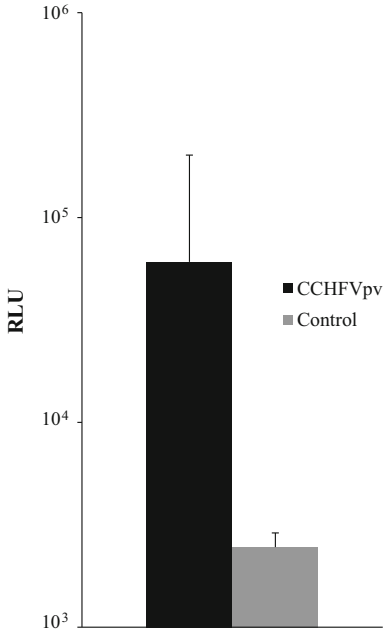

Fig. 1 The CCHFVpv/Luc titer. To generate a pseudotype virus, 293T cells were transfected with pC-CCHFV GP del10 or pC-GFP as a control. After overnight incubation, the cells were infected with VSVΔG/Luc-*G. The collected supernatants were incubated with Vero cells to determine the titer of the pseudotype virus. Luciferase activity was measured as described in Materials and methods at 1 day postinfection. The tests were performed in triplicate and means ± standard deviations are shown. RLU, relative luciferase units. "Control" indicates the titer of the pseudotype virus, which was generated using pC-GFP

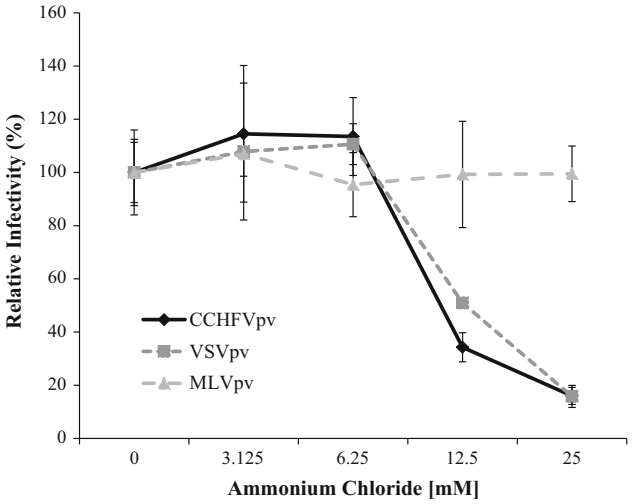

Fig. 2 pH dependence of CCHFVpv entry. VeroE6 cells were pretreated with NH_4Cl for 60 min at the designated concentrations before infection with CCHFVpv/Luc, VSVpv/Luc, or MLVpv/Luc. After 1 day, luciferase activity was measured as described in Materials and methods. The tests were performed in triplicate, and means ± standard deviations are shown

virus envelope protein) were generated as described previously [22].

Reporter assay

To quantitatively measure the infectivity of CCHFVpv, the reporter activity in inoculated cells was assayed at 1 day postinfection as follows: For the pseudotype virus with the luciferase gene, luciferase activity was measured using a Bright-Glo luciferase assay system (Promega) in accordance with the manufacturer's protocol with GloMax (Promega). For the pseudotype virus with the GFP gene, the supernatant was replaced with PBS (-), and GFP-positive cells were counted under a fluorescence microscope (BZ-X710, KEYENCE). The infectious units (IU) were determined as the number of the GFP-positive cells.

Effects of ammonium chloride (NH_4Cl) and methyl-β-cyclodextrin (MβCD) on infectivity

Cells were treated with the indicated concentrations of NH_4Cl for 60 min or MβCD for 30 min at 37 °C and then inoculated with the pseudotype viruses in DMEM supplemented with 2 % (v/v) FBS. Infectivity was determined as described above. To restore cholesterol, cells were treated with 200 μM exogenous cholesterol for 30 min after cholesterol removal.

Serum samples

The CCHF patient serum sample was obtained from the Center for Disease Control and Prevention (Atlanta, Georgia, USA). Two serum samples, which were collected from healthy Japanese adults, were used as controls. The usage of the sera was approved by the Research and Ethics Committee of the National Institute of Infectious Diseases, Tokyo, Japan (reference no. 439).

Neutralization assay

CCHFVpv was incubated with diluted human serum samples for 30 min at 37 °C and inoculated onto VeroE6 cells. At 1 day after inoculation, CCHFVpv infectivity was measured as described above.

Flow cytometry

Antibody staining and analysis were performed as described previously [32, 36]. The antibodies used to detect lectins were as follows: normal mouse IgG antibody (Mouse IgG Isotype Control) (R&D systems), anti-fCD2 antibody [37], anti-DC-SIGN antibody (Clone DC28, R&D systems), and anti-LSECtin antibody (SOTO-1, Santa Cruz Biotechnology, Inc.).

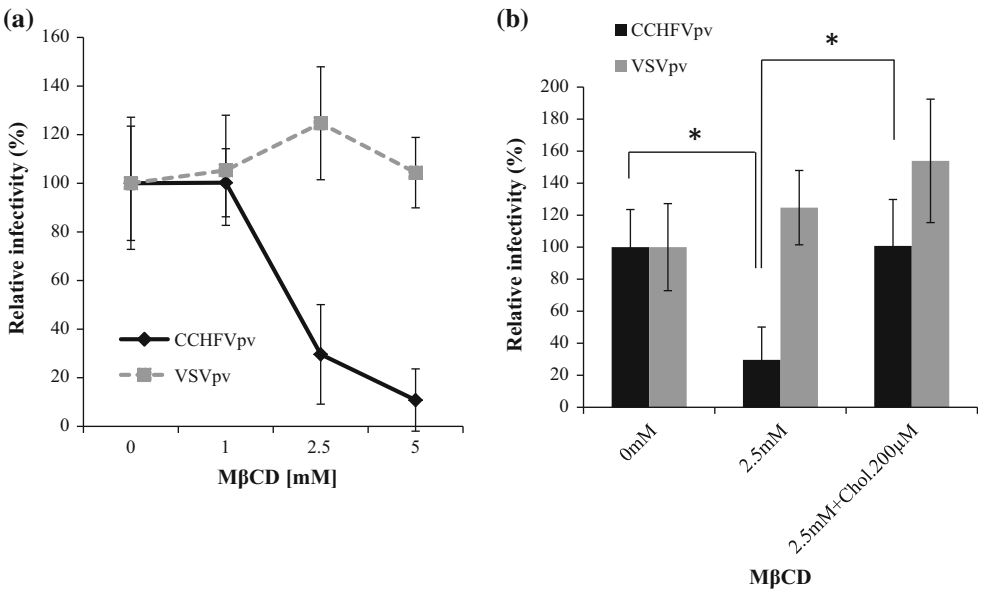

Fig. 3 Cholesterol dependence of CCHFVpv entry. (a) VeroE6 cells were pretreated with various concentrations of MβCD for 30 min before infection with CCHFVpv/Luc or VSVpv/Luc. After 1 day, luciferase activity was measured as described in Materials and methods. (b) VeroE6 cells were depleted of cholesterol and infected with pseudotype viruses or cholesterol was depleted and restored before infection in the presence of MβCD or cholesterol. The tests were performed in triplicate, and means ± standard deviations are shown. *, statistically significant ($P < 0.05$)

Statistical analysis

The differences in the infectivity were compared using Student's *t*-test.

Results

Effect of deletions in the GP carboxyl terminal region on the titer of the pseudotype virus

To generate pseudotyped VSV bearing the CCHFV envelope GP, we first used the full-length GP as an envelope protein and VSVΔG/GFP-*G, the reporter gene of which is GFP, as a seed virus. VeroE6 cells were inoculated with the resultant pseudotype virus and the cells expressing GFP were counted. The titer of the pseudotype virus with full-length GP was 200 IU/ml (Table 1). Next, we examined the effects of deletions within the carboxyl terminal region of GP on the titers of the pseudotype virus, because deletion of the Gc cytoplasmic tail might change the trafficking of the envelope protein, which could affect pseudotype virus production. Furthermore, it was reported previously that a pseudotyped VSV bearing the envelope protein of SARS-CoV with a truncation in the cytoplasmic domain was more efficiently incorporated in the viral particle than the full-length protein [20]. We used four mutant GPs (Table 1) as envelope proteins to generate the pseudotype virus. All of the cytoplasmic-region-deleted GPs that we examined produced higher titers of the pseudotype virus than the full-length GP (Table 1). The highest titer, which was approximately 30 times that of the full-length GP, was obtained with the GP del10 mutant. The pseudotype virus with GP del10, named CCHFVpv, was used for subsequent experiments. Consistent results were obtained when a luciferase reporter was used instead of a GFP reporter (Fig. 1). The luciferase activity obtained with CCHFVpv/Luc was approximately 30 times higher than in the cells incubated with the pseudotype virus without the envelope protein.

Characteristics of CCHFVpv infection

We examined whether CCHFVpv infection had the characteristics that were recognized in authentic CCHFV infection. Authentic CCHFV infection has been reported to be dependent on pH and cholesterol [14, 15]. First, we examined the effects of NH_4Cl, which increases the pH of intracellular compartments, on CCHFVpv infection. As shown in Fig. 2, treatment with NH_4Cl at concentrations of 12.5 mM and 25 mM decreased CCHFVpv/Luc infection in a dose-dependent manner. NH_4Cl treatment had a similar effect on infection with VSVpv/Luc, which requires a low-pH step for the entry [15, 38]. In contrast, the treatment did not affect the infectivity of MLVpv/Luc, which is independent of pH for the entry [22]. This indicated that CCHFVpv infection was pH dependent. Next, the effects of MβCD, which removes cholesterol from the cell membranes, were investigated. As shown in Fig. 3a, treatment with MβCD at concentrations of 2.5 mM and 5 mM decreased CCHFVpv/Luc infection in a dose-dependent manner. As shown in Fig. 3b, MβCD treatment decreased CCHFVpv/Luc infection, and the inhibitory effects were reduced when the cholesterol was restored by adding exogenous cholesterol after the removal of cholesterol. This indicated that CCHFVpv infection was cholesterol dependent. Furthermore, to confirm the dependence of

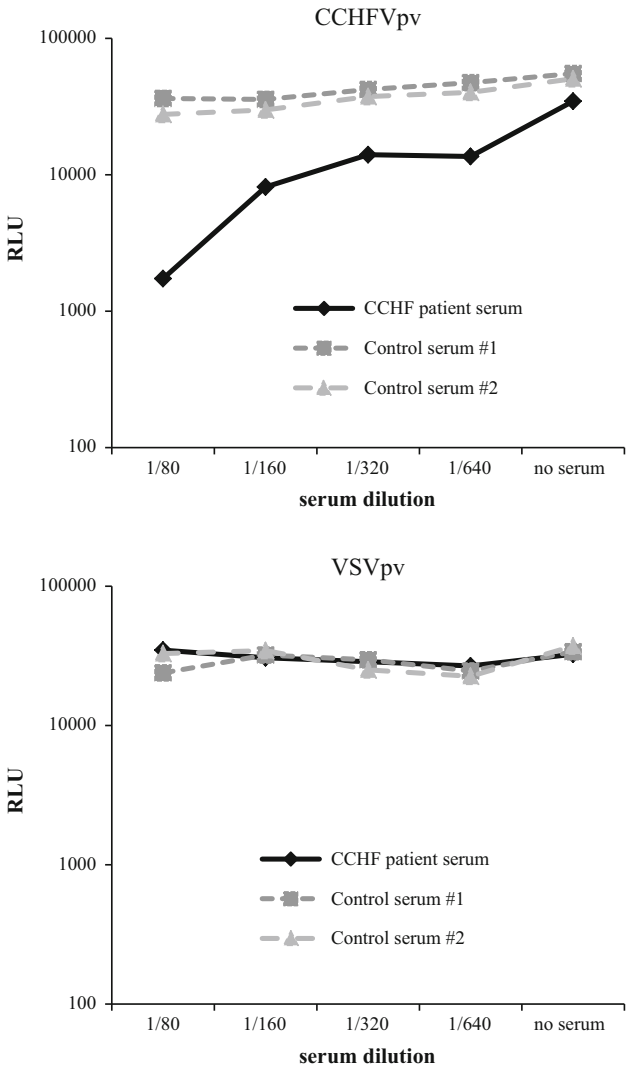

Fig. 4 Neutralization of CCHFVpv and VSVpv with serum from a CCHF patient. A neutralization assay was performed by incubating CCHFVpv/Luc or VSVpv/Luc with the CCHF patient's serum or sera collected from two healthy Japanese CCHFV-antibody-negative adults at the designated dilutions for 30 min. The mixtures were added to VeroE6 cells, and luciferase activity was measured at 1 day postinfection. The tests were performed in duplicate, and means are shown. RLU, relative luciferase units

CCHFVpv infection on the CCHFV envelope GP, we used sera obtained from healthy donors and a CCHF patient in the convalescent phase. As shown in Fig. 4, the CCHF patient serum inhibited infection with CCHFVpv/Luc, but not with VSVpv/Luc. In contrast, the control sera from the two healthy subjects did not affect the infection with either of the two pseudotype viruses. These results indicated that CCHFVpv infection was dependent on the CCHFV envelope GP. These results suggest that the entry mechanism of CCHFVpv has characteristics similar to those of authentic CCHFV. This pseudotype is therefore likely to be useful for investigating the entry mechanism of CCHFV.

Cell-surface molecules involved in CCHFV infection

Because the CCHFVpv titer measured in Jurkat cells was found to be quite low (5 IU/mL) in comparison to that in VeroE6 cells (6250 IU/mL) (Table 1), Jurkat cells were used to investigate the involvement of C-type lectins, cell-surface molecules that have been well investigated in studies of various viral entry mechanisms and shown to enhance the entry of Ebola virus and Lassa virus entry into cells [32, 36]. Expression of a control molecule (fCD2ΔCT) and C-type lectins was confirmed by flow cytometry (Fig. 5a). As shown in Fig. 5b, CCHFVpv/GFP and Lassapv/GFP infections in Jurkat cells were enhanced by the expression of DC-SIGN. Whereas LSECtin expression enhanced Lassapv/GFP and EBOVpv/GFP infection (Fig. 5b), the lectin did not affect the CCHFVpv/GFP infection. The results showed that DC-SIGN, but not LSECtin, was involved in CCHFVpv infection. It was reported previously that the binding of Lassapv to DC-SIGN, which mainly recognizes high-mannose-type glycans, was blocked by mannan, a polymer of mannose [32]. CCHFVpv/GFP infection in DC-SIGN-expressing Jurkat cells was inhibited by pretreatment with mannan, but not with GlcNAcβ1-2Man, one of the high-affinity ligands of LSECtin [32, 39] (Fig. 5c). Furthermore, CCHFVpv/GFP infection was also inhibited by pretreatment with the antibody to DC-SIGN (Fig. 5d). These results show that CCHFVpv may preferentially infect cells that express DC-SIGN as a result of binding between carbohydrates on the envelope GP and DC-SIGN.

Discussion

Quantitative measurement of infection with a pseudotyped VSV in which the viral envelope protein has been replaced with that of a different virus is highly sensitive, rapid, and easy. Here, we report the creation of a VSV pseudotype bearing the envelope protein of CCHFV, which requires high BSL containment for experimental use. Although a pseudotyped VSV bearing a full-length CCHFV envelope GP has been reported recently [40], the intensity of luminescence (the expression of the reporter used in the experiment) in infected cells was only eight times higher than the background. Therefore, a pseudotype virus with a much higher titer is needed for research and diagnosis. We found that the use of CCHFV GP with a truncated carboxyl terminus resulted in a higher titer of pseudotyped VSV (30 times higher than background) (Fig. 1). Although the precise reason(s) why the truncation of the carboxyl terminus of CCHFV GP produced high viral titers is unclear, the altered localization of Gn and Gc and/or proper assembly with the VSV proteins may have been involved.

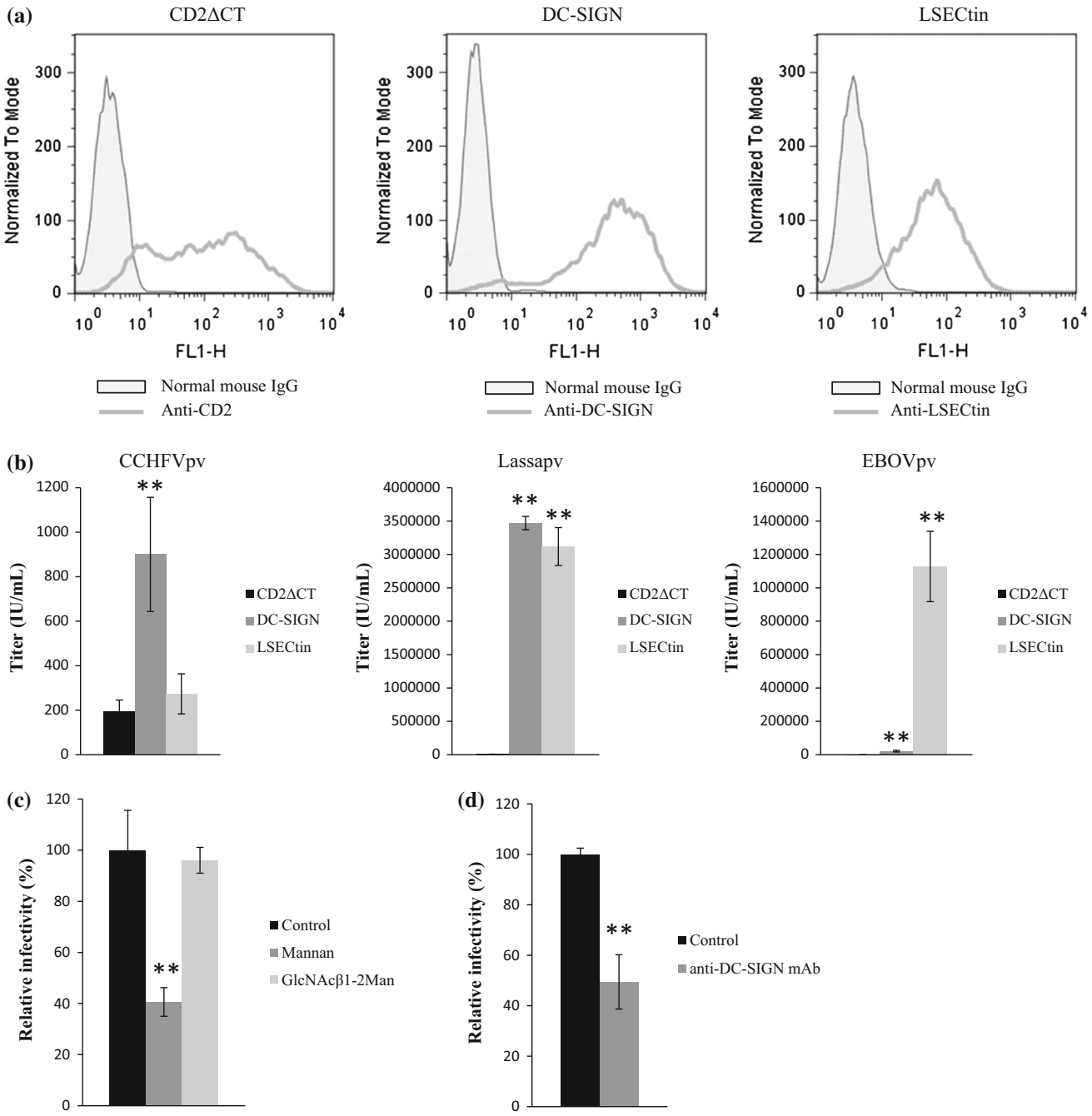

Fig. 5 Role of C-type lectin as an entry factor for CCHFVpv. (a) The expression of fCD2ΔCT and C-type lectins was confirmed by flow cytometry. (b) Jurkat cells expressing DC-SIGN or LSECtin were infected with CCHFVpv/GFP, Lassapv/GFP, or EBOVpv/GFP. After overnight incubation, the GFP-expressing fluorescent cells were counted. (c) Jurkat cells expressing DC-SIGN were treated with mannan or GlcNAcβ1-2Man and then infected with CCHFVpv/GFP. After overnight incubation, the GFP-expressing fluorescent cells were counted. (d) Jurkat cells expressing DC-SIGN were pretreated with anti-DC-SIGN antibody (MAB161, R&D systems) or normal mouse IgG as a control and then infected with CCHFVpv/GFP. After overnight incubation, the GFP-positive cells were counted. The tests were performed in triplicate, and means ± standard deviations are shown. **, statistically significant ($P < 0.01$) when compared with data from fCD2ΔCT (b) or controls (c and d)

Nonetheless, because of its high titer and characteristics that were recognized in authentic CCHFV in terms of the pH- and cholesterol dependence of infection (Table 1, Figs. 2 and 3), the pseudotyped VSV with truncated CCHFV GP (e.g., del10 mutant in Table 1) is potentially useful for studies on the entry mechanisms of CCHFV and serosurveillance experiments, such as the measurement of neutralizing activities. As CCHFV requires high biosafety

containment, this pseudotype virus will be a powerful tool for studying CCHFV.

Because the CCHFV envelope protein is a glycoprotein [10, 35] and some phleboviruses, such as Rift Valley fever virus, Uukuniemi virus [33], and SFTSV [34], used a C-type lectin, DC-SIGN, as a receptor, we expected C-type lectins to be receptors for CCHFV. In this study, we found that CCHFVpv efficiently infected DC-SIGN-expressing cells, although we examined only two C-type lectins (Fig. 5), indicating that DC-SIGN is a possible novel entry factor for CCHFV. The result suggests that the role of DC-SIGN in cell entry is conserved among members of the family *Bunyaviridae*. CCHFV initially replicates in dendritic cells and tissue resident macrophages, and the virus then migrates to a broad range of tissues and organs, including regional lymph nodes, spleen and liver [6, 41, 42]. As DC-SIGN is present on the surface of dendritic cells [28, 43] and macrophages [44], the results of this study agree with the tropism of this virus and suggest that DC-SIGN has important role in the infection of dendritic cells and macrophages. However, as the inhibitory effect by mannan or anti-DC-SIGN antibody was only approximately 50 % (Fig. 5c and d), the mechanism of enhancement of CCHFVpv infection by DC-SIGN might be nonspecific or could involve interactions other than those with the carbohydrate-recognition domain of DC-SIGN.

The effects of two C-type lectins on CCHFVpv infection differed from the effects on EBOVpv and Lassapv infection (Fig. 5b). CCHFVpv infection was enhanced by DC-SIGN but not by LSECtin. In contrast, Lassapv infection was enhanced by both lectins, and EBOVpv infection was enhanced to a much greater extent by LSECtin than by DC-SIGN. Such different features suggest that the glycosylation status of the CCHFV envelope GP is different from that of the Ebola and Lassa virus GPs. However, there are several possible reasons why LSECtin expression does not enhance CCHFVpv infection. For example, it is possible that the molecule does not bind the CCHFVpv particle, resulting in no involvement (no enhancement) in the viral infection. A second possibility is that the molecule binds the CCHFVpv particle but that the binding does not result in enhancement of infection due to inadequate interaction with a putative factor or cofactor necessary for infection. A third possibility is that binding of the CCHFVpv particle with LSECtin enhances transport to a degradation pathway.

There is a possibility that other molecules that have not yet been examined may enhance CCHFV infection more than DC-SIGN. To investigate the entry mechanism of CCHFV more precisely, further experiments will be needed including examination of other C-type lectins (such as mannose receptor, L-SIGN, or langerin), identification of still unknown receptors, and binding and internalization assays. In addition, candidate molecules/pathways that are eventually found to be involved in CCHFVpv entry into cells should be studied using wild-type CCHFV, because it is unclear whether the entry mechanisms of CCHFVpv and CCHFV are completely identical. Nevertheless, this pseudotype virus can provide novel insights into the mechanism of CCHFV infection and can be developed as a surrogate model for CCHFV research.

Acknowledgements This work was supported in part by grants for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor, and Welfare of Japan (H25-Shinko-Ippan-004) and by Grants-in-Aid for Exploratory Research (26660227, 25670222) from the Japan Society for the Promotion of Science (JSPS).

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