

Short Communication

Vesicular stomatitis virus pseudotyped with severe acute respiratory syndrome coronavirus spike protein

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Severe acute respiratory syndrome coronavirus (SARS-CoV) contains a single spike (S) protein, which binds to its receptor, angiotensin-converting enzyme 2 (ACE2), induces membrane fusion and serves as a neutralizing antigen. A SARS-CoV-S protein-bearing vesicular stomatitis virus (VSV) pseudotype using the VSVΔG^{*} system was generated. Partial deletion of the SARS-CoV-S protein cytoplasmic domain allowed efficient incorporation into VSV particles and led to the generation of a pseudotype (VSV-SARS-St19) at high titre. Green fluorescent protein expression was demonstrated as early as 7 h after infection of Vero E6 cells with VSV-SARS-St19. VSV-SARS-St19 was neutralized by anti-SARS-CoV antibody and soluble ACE2, and its infection was blocked by treatment of Vero E6 cells with anti-ACE2 antibody. These results indicated that VSV-SARS-St19 infection is mediated by SARS-CoV-S protein in an ACE2-dependent manner. VSV-SARS-St19 will be useful for analysing the function of SARS-CoV-S protein and for developing rapid methods of detecting neutralizing antibodies specific for SARS-CoV infection.

Received 9 February 2005
Accepted 21 April 2005

Severe acute respiratory syndrome (SARS) is a recently described infectious disease caused by a newly identified coronavirus, SARS-CoV (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003). With a mortality rate of over 9%, SARS has had major health and socio-economic impacts (Fouchier *et al.*, 2003). Despite intensive efforts, no effective antiviral treatments against SARS have yet been established. Studies of SARS-CoV infection have been limited because of the highly infectious nature of the virus and the problem of recent cases in which infection was suspected to have occurred in research laboratories.

Entry of SARS-CoV into susceptible cells is mediated by binding of the viral spike (S) protein to receptor molecules. The SARS-CoV-S protein has a 13 aa signal peptide at its N terminus, a single ectodomain of 1182 aa and a transmembrane region followed by a cytoplasmic domain of 28 aa (Marra *et al.*, 2003; Rota *et al.*, 2003). Recently, pseudotyped retroviruses bearing SARS-CoV-S protein have been generated by several laboratories (Hofmann *et al.*, 2004; Nie *et al.*, 2004; Simmons *et al.*, 2004). It has been shown that these pseudotyped viruses have a cell tropism identical to SARS-CoV and that their infection is dependent on a receptor molecule, angiotensin-converting enzyme 2

(ACE2), indicating that infection is mediated solely by SARS-CoV-S protein. Pseudotyped viruses provide a safe viral entry model because of their inability to produce infectious progeny virus. A quantitative assay of pseudotyped virus infection could facilitate research on SARS-CoV entry, cell tropism and neutralizing antibodies. Interestingly, it was reported that a pseudotyped retrovirus bearing a SARS-CoV-S protein variant with a truncation in the cytoplasmic domain was incorporated more efficiently into retrovirus particles than the full-length S protein (Giroglou *et al.*, 2004; Moore *et al.*, 2004).

Another pseudotyping system with a vesicular stomatitis virus (VSV) particle [the VSVΔG^{*} system, in which the VSV G gene is replaced by the green fluorescent protein (GFP) gene] was reported previously to produce pseudotype VSV particles incorporating the envelope glycoproteins of several RNA viruses (i.e. measles virus, hantavirus, Ebola virus or hepatitis C virus; Matsuura *et al.*, 2001; Ogino *et al.*, 2003; Takada *et al.*, 1997; Tatsuo *et al.*, 2000). This system may be useful for research on viral envelope glycoproteins due to the ability of the pseudotype to grow at high titres in a variety of cell lines. The pseudotype virus titre obtained with the VSVΔG^{*} system is generally higher than that of the pseudotype retrovirus system (Ogino *et al.*, 2003). Furthermore, infection of target cells with pseudotype VSV can be readily detected as GFP-positive cells by 16 h

Detection of expression of SARS-CoV-S protein and soluble mutated ACE2 are available as supplementary material in JGV Online.

post-infection (p.i.) because of the high level of GFP expression in the VSV Δ G* system (Ogino *et al.*, 2003). In contrast, the time required for infection in the pseudotype retrovirus system is 48 h (Moore *et al.*, 2004; Nie *et al.*, 2004), which is similar to the time required for SARS-CoV to replicate to a level that results in plaque-forming or cytopathic effects in infected cells. To date, there have been no reports of VSV pseudotyped with the S protein of a coronavirus. Pseudotyping of SARS-CoV-S protein using the VSV Δ G* system may have advantages for studying the functions of SARS-CoV-S protein, as well as for developing a rapid detection system to examine neutralizing antibodies specific for SARS-CoV infection.

To generate VSV pseudotyped with SARS-CoV-S protein, we first constructed an expression plasmid encoding full-length SARS-CoV-S protein. The cDNA of SARS-CoV-S protein was amplified using forward primer S-Bam-f (5'-GGATCCAAGTGATATTCTTGTTAACAAC-3') and reverse primer S-Bam-r (5'-GGATCCAAGAGTAAAAA-TCTCATAAAC-3') and cloned into the expression vector pKS336 (Saijo *et al.*, 2002). The resulting plasmid, pKS-SARS-S, was transfected into 293T cells (see Supplementary Fig. S1, available in JGV Online), followed by infection with VSV Δ G* (Matsuura *et al.*, 2001). When the culture supernatants of the infected 293T cells were inoculated on to Vero E6 cells, commonly used for SARS-CoV propagation, only small numbers of GFP-expressing cells were observed (data not shown). This result indicated that the VSV pseudotype bearing the full-length SARS-CoV-S protein was not very infectious. Next, we generated an expression plasmid encoding a C-terminal-truncated version of the SARS-CoV-S protein. The cDNA of C-terminal-truncated SARS-CoV-S protein was amplified from pKS-SARS-S using forward primer S-Bam-f and reverse primer S-Bam19r (5'-GGG-ATCCTTAGCAGCAAGAACCACAAGAGCATG-3'), followed by cloning into pKS336. The resulting plasmid, pKS-SARS-St19, encoded the full-length SARS-CoV-S protein except for the C-terminal 19 aa. When 293T cells were transfected with the expression plasmid pKS-SARS-St19, expression of the S protein was detected on the cell membrane (Supplementary Fig. S1). In contrast, transfection of the plasmid pKS-SARS-St19rev, in which the cDNA of C-terminal truncated SARS-CoV-S protein was inserted in the reverse orientation, did not show expression of SARS-CoV-S protein and was therefore used as a negative control for generating the VSV pseudotype. To generate a VSV pseudotype with the C-terminal truncated SARS-CoV-S protein, we inoculated VSV Δ G* on to 293T cells transfected with either pKS-SARS-St19 or pKS-SARS-St19rev. VSV Δ G*-G was used as a positive control, in which the deleted VSV-G protein was provided *in trans* encoded by pCAG-VSV-G (a kind gift from Dr Y. Matsuura, Osaka University, Japan) (Fig. 1a). The SARS-CoV-S protein-bearing VSV pseudotype, referred to as VSV-SARS-St19, obtained from 293T cells transfected with pKS-SARS-St19, efficiently infected Vero E6 cells (Fig. 1a). The titre of VSV-SARS-St19 was 5.0×10^5 infectious units (IU) ml⁻¹. Since

partial deletion of the SARS-CoV-S protein cytoplasmic domain allowed efficient incorporation into VSV particles and led to the generation of pseudotype at high titre, it was suggested that the intact cytoplasmic domain of SARS-CoV-S protein may have interrupted correct assembly of the pseudotype particles.

In order to confirm that the SARS-CoV-St19 protein was indeed incorporated into VSV particles, VSV pseudotypes were purified by ultracentrifugation using an RPS40T rotor (Hitachi) at 35 000 r.p.m. for 1 h through 20% sucrose and then analysed by Western blotting using a rabbit antibody specific for aa 1124–1140 of SARS-CoV-S protein (Imgenex), a rabbit anti-VSV-G antibody (a kind gift from Dr S. Nagata, Tokyo University, Japan) and a mouse monoclonal antibody specific for VSV-M (a kind gift from Dr M. A. Whitt, GTx, Inc., TN, USA) (Fig. 1b). The VSV-M and SARS-CoV-S proteins were detected in VSV-SARS-St19, whereas the VSV-M and -G protein were detected in VSV Δ G*-G. These results indicated that the SARS-CoV-St19 protein expressed in 293T cells was incorporated efficiently into VSV particles.

To determine the optimal incubation period for detection of VSV-SARS-St19 infection, we performed a time-course analysis of GFP expression. Vero E6 monolayers on 24-well glass slides were infected with VSV-SARS-St19. At various time points p.i., cells were photographed under a fluorescent microscope. The number of GFP-expressing cells in the photographs was counted using ImageJ software (<http://rsb.info.nih.gov/ij/>). Interestingly, GFP expression was detected clearly at 7 h p.i., although the fluorescence intensity of GFP expression was increased at 9 h p.i. (Fig. 1c). Infected cells detached from culture slides, presumably due to a cytopathic effect of VSV proteins, after incubation for 16 h (data not shown). As pseudoviruses are unable to produce progeny viruses, this system would be useful for the evaluation of cell entry mechanisms mediated by SARS-CoV-S protein. Therefore, the infection efficiency of VSV-SARS-St19 can be evaluated without incubation for up to 16 h p.i. Time-course analysis of the number of GFP-positive cells showed that quantification of VSV-SARS-St19 infection was possible at 7 h p.i. (Fig. 1c). Therefore, we counted the number of GFP-positive cells infected with VSV-SARS-St19 at 7 h p.i. for further study.

To confirm the specificity of infection, a rabbit anti-SARS-CoV antibody was tested for its ability to neutralize VSV-SARS-St19. The antibody was raised against UV-inactivated, purified SARS-CoV and reacted with SARS-CoV-S protein in an immunofluorescence assay (see Supplementary Fig. S1); the antibody showed neutralizing activity against SARS-CoV infection on Vero E6 cells at a dilution of 1 : 2560 (data not shown). As a negative control, we used a rabbit antibody raised against SARS-CoV-N protein-specific oligopeptides (Mizutani *et al.*, 2004). Culture medium containing 500 IU VSV-SARS-St19 was pre-incubated with serially diluted antibodies followed by inoculation on to Vero E6 cells. Pre-incubation with anti-SARS-CoV antibody showed

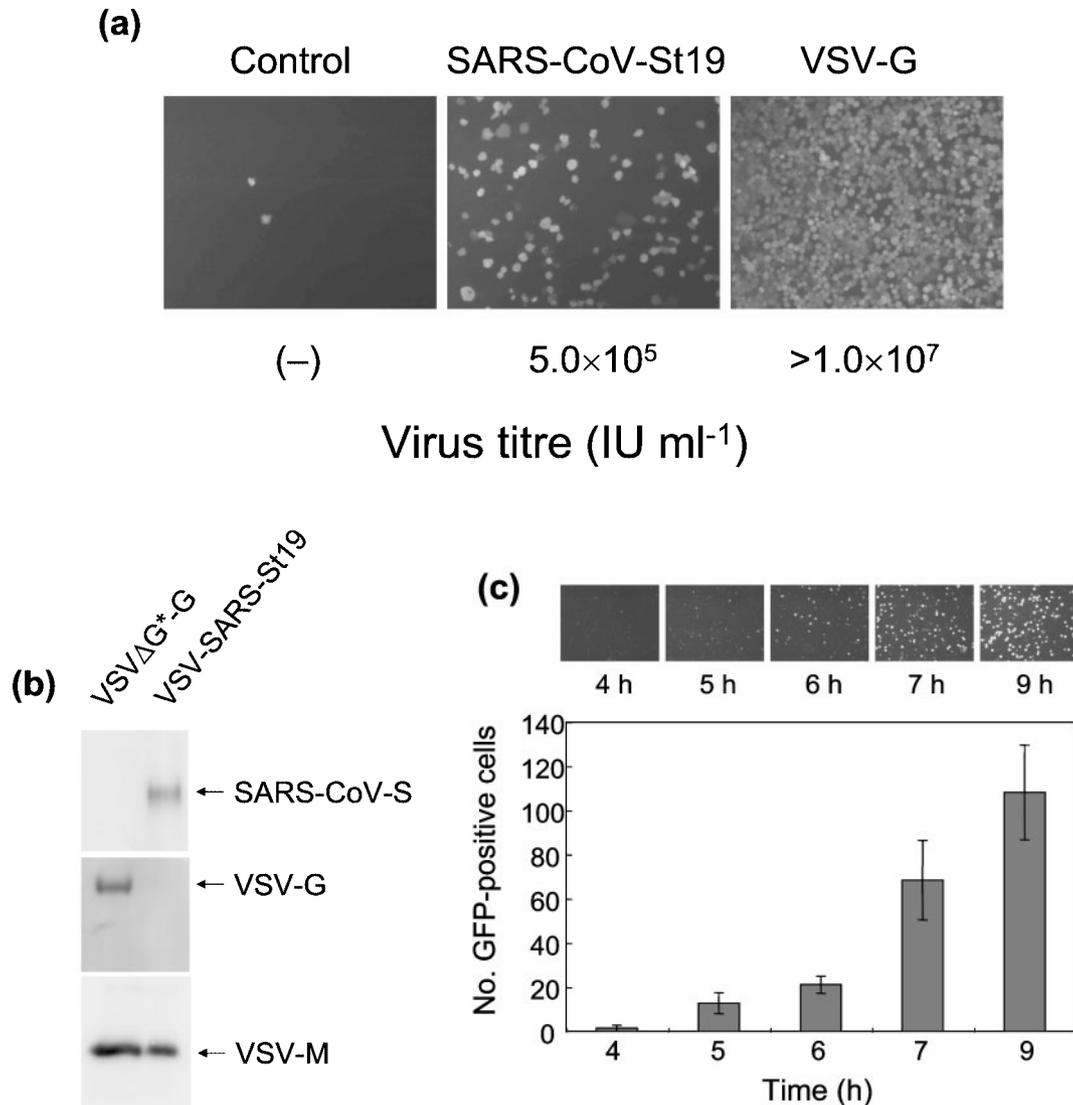


Fig. 1. Generation and characterization of VSV pseudotyped with SARS-CoV-S protein. (a) VSVΔG⁺ was inoculated on to 293T cells expressing the indicated glycoproteins. After 24 h, culture supernatants were collected, filtered through a 0.22 μm pore size filter and inoculated on to Vero E6 cells. GFP expression was examined under a fluorescent microscope. The titre of pseudotype viruses was determined by end-point dilution. (b) Incorporation of SARS-CoV-S protein into pseudotype VSV particles. VSV-SARS-St19 and VSVΔG⁺-G were partially purified by ultracentrifugation through 20% sucrose. Viral proteins were analysed by Western blotting. The bands of SARS-CoV-S protein (180 kDa), VSV-G protein (62 kDa) and VSV-M protein (32 kDa) are indicated. (c) Rapid detection and quantification of VSV-SARS-St19 infection. Vero E6 cells were infected with VSV-SARS-St19 and GFP expression was examined at the indicated time points p.i. under a fluorescent microscope. The bar graph represents the number of GFP-positive cells per microscopic field (mean ± SD) calculated from six fields.

a significant reduction in the titre of VSV-SARS-St19 in a dose-dependent manner (Fig. 2a). Fifty per cent neutralizing activity was calculated at a dilution of 1:2000. In contrast, infection of Vero E6 cells with VSVΔG⁺-G was not affected by the anti-SARS-CoV antibody, even at a lower dilution (1:80 dilution) (Fig. 2a). When VSV-SARS-St19 was pre-incubated with control anti-SARS-CoV-N antibody, no neutralizing activity was observed (Fig. 2a). The

possibility of carry-over of VSV-G during the production of VSV-SARS-St19, leading to infection of Vero E6 cells, was excluded, since an anti-VSV-G monoclonal antibody (P2F3, a kind gift from Dr. S. Nagata), which showed specific neutralizing activity against VSV infection (Nagata *et al.*, 1992), had no effect on infection of Vero E6 cells by VSV-SARS-St19 (Fig. 2b), but prohibited infection by VSVΔG⁺-G. These results indicated that infection of Vero E6 cells with

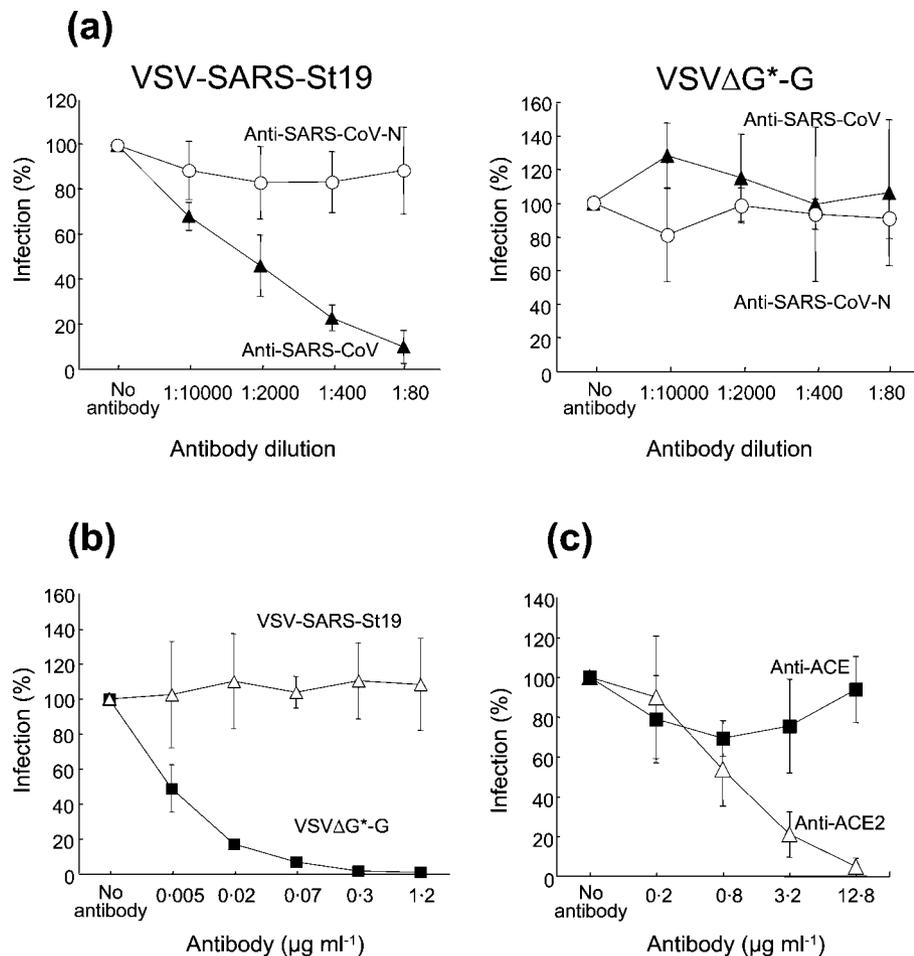


Fig. 2. Specificity of VSV-SARS-St19 pseudotype infection. (a) VSV-SARS-St19 (left) or VSVΔG*-G (right) was pre-incubated with serially diluted anti-SARS-CoV (▲) or anti-SARS-CoV-N (○) followed by inoculation on to Vero E6 cells. (b) VSV-SARS-St19 (△) or VSVΔG*-G (■) was pre-incubated with serially diluted anti-VSV-G antibody. (c) Vero E6 cells were pre-incubated with serially diluted anti-ACE2 (■) or anti-ACE2 (△) antibody and infected with VSV-SARS-St19. At 7 h p.i., GFP-positive cells were counted. The number of GFP-positive cells in the absence of antibodies was set as 100%. The results are shown as mean ± SD for at least three independent assays.

VSV-SARS-St19 was mediated solely by SARS-CoV-S protein.

To determine whether anti-ACE2 antibody could inhibit VSV-SARS-St19 infection, Vero E6 cells were pre-incubated with anti-ACE2 antibody (R&D Systems) and the infectivity of VSV-SARS-St19 was examined. As a negative control, cells were pre-incubated with anti-ACE antibody (R&D Systems). As shown in Fig. 2(c), pre-incubation with anti-ACE2 antibody resulted in a significant reduction in VSV-SARS-St19 infection in a dose-dependent manner. Anti-ACE2 antibody, at a concentration of 12.8 μg ml⁻¹, completely inhibited VSV-SARS-St19 infection. In contrast, anti-ACE antibody had no appreciable effect on infection. These results indicated that VSV-SARS-St19 infection is ACE2-dependent. These observations were consistent with those reported previously by Li *et al.* (2003); on SARS-CoV infection of Vero E6 cells, the same polyclonal antibody

showed an inhibitory effect on cytopathicity in a dose-dependent manner and complete inhibition was observed at an anti-ACE2 antibody concentration of approximately 10 μg ml⁻¹.

We further analysed whether soluble ACE2 protein could inhibit infection by VSV-SARS-St19. We introduced two amino acid substitutions at aa 374 and 378 within the putative catalytic domain of ACE2 (soACE2-NN; Supplementary Fig. S2). Purified soACE2-NN protein expressed in a baculovirus expression system was pre-incubated with VSV-SARS-St19 or VSVΔG*-G and the mixtures inoculated on to Vero E6 cells. As shown in Fig. 3(a), soluble ACE2 protein strongly inhibited VSV-SARS-St19 infection in a dose-dependent manner, but did not affect VSVΔG*-G infection. These observations indicated that purified soluble ACE2 specifically inhibited VSV-SARS-St19 infection. The highest concentration of soACE2 showed partial inhibition

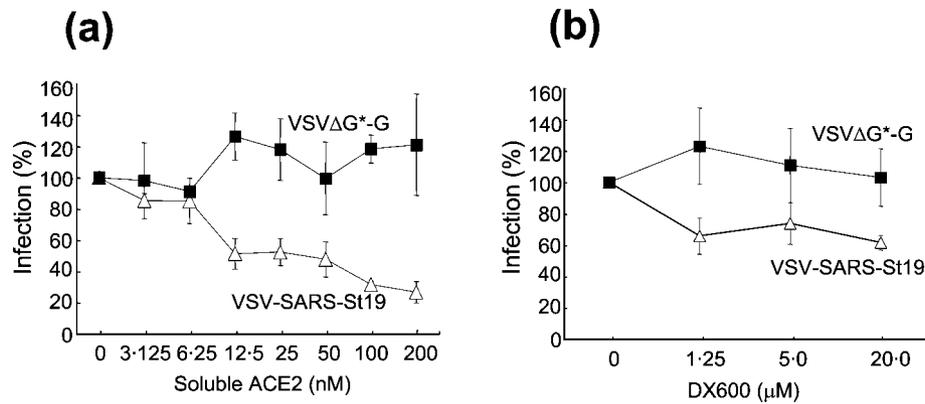


Fig. 3. Inhibition of VSV-SARS-St19 infection. VSV-SARS-St19 (Δ) or VSV Δ G*-G (\blacksquare) was pre-incubated with serially diluted soACE2NN (a) or DX600 (b), followed by inoculation on to Vero E6 cells. The infectivity of the pseudotypes was examined as described in Fig. 2.

(70–80%) of VSV-SARS-St19 infectivity. However, the possibility that ACE2-independent entry of the pseudotype virus also existed could be excluded, since anti-ACE2 antibody completely inhibited VSV-SARS-St19 infectivity and almost all of the infection was dependent on ACE2 (Fig. 2c). The amount of soluble ACE2 that neutralized 50% of the VSV-SARS-St19 infection (50% neutralizing dose; ND₅₀) was estimated to be 12.5 nM. The neutralization activity of soACE2 in the present study was somewhat lower than that reported by Moore *et al.* (2004). This may have been due to the different strategies used for soACE2 preparation. The neutralization activity of soACE2 against SARS-CoV infection was lower than that of soluble mouse hepatitis virus receptor (soMHVR) against MHV infection (ND₅₀ = 1 nM; Miura *et al.*, 2004; Zelus *et al.*, 1998). These observations suggested that SARS-CoV infection is regulated less strictly by the receptor function than MHV infection. Alternatively, a subdomain of ACE2 protein might represent increasing neutralization activity if bound to the SARS-CoV-S protein more strongly than the full-length protein. Indeed, neutralization of MHV-A59 infection was enhanced when the N-terminal subdomain of MHVR was used for neutralization experiments (Zelus *et al.*, 1998). It is noteworthy that an alternative splice variant of mouse ACE2 mRNA, consisting of the 5' half of mouse ACE2 mRNA and not containing the metalloproteinase catalytic domain, has been reported (Komatsu *et al.*, 2002).

We then investigated whether a known ACE2-specific peptide inhibitor competed against ACE2-mediated pseudotype virus infection. VSV-SARS-St19 was pre-incubated with various concentrations of DX600 (Phoenix Pharmaceuticals), which has been shown to bind to and inhibit the enzymic activity of ACE2 (Huang *et al.*, 2003), and the mixture was then inoculated on to Vero E6 cells. Interestingly, DX600 inhibited VSV-SARS-St19 infection, but did not inhibit VSV Δ G*-G infection. Higher concentrations (>1.25 μ M) of DX600 were required for 30–40% inhibition, indicating that this inhibition was weak

(Fig. 3b). It has been shown that enzymic activity is not required for ACE2 protein as a SARS-CoV receptor (Li *et al.*, 2003). However, our results indicated that incubation of DX600 partially influenced the ACE2 function as a SARS-CoV receptor. Further investigation including inhibition studies with live SARS-CoV will be needed to elucidate the efficacy of DX600. Our results suggest that ACE2-binding peptides can be used as specific inhibitors of SARS-CoV-S protein-mediated infection. Based on the results of neutralization experiments using anti-SARS-CoV antibody and anti-ACE2 antibody, we conclude that VSV-SARS-St19 infection of target cells is mediated by SARS-CoV-S protein. The assay system described here would be useful not only for developing a safe and rapid method of detecting neutralizing antibodies to SARS-CoV, but also for screening of inhibitors of SARS-CoV-S protein-mediated infection.

Acknowledgements

We thank Dr M. A. Whitt for providing the VSV Δ G* and anti-VSV-M antibody, Dr S. Nagata for providing monoclonal antibody P2F3 and rabbit anti-VSV G antibody, and Dr Y. Matsuura for providing pCAG-VSVG. We also thank Ms M. Ogata for her assistance. This work was supported in part by a grant-in-aid from the Ministry of Health, Labor and Welfare of Japan and the Japan Health Science Foundation, Tokyo, Japan.

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