

Research Communication

A Convenient Cell Fusion Assay for the Study of SARS-CoV Entry and Inhibition

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Summary

SARS-CoV spike (S) protein-mediated cell fusion is important for the viral entry mechanism and identification of SARS-CoV entry inhibitors. In order to avoid the high risks involved in handling SARS-CoV and to facilitate the study of viral fusion mechanism, we established the cell lines: SR-COS7 cells that stably express both SARS-CoV S protein and red fluorescence protein, R-COS7 cells that stably express red fluorescence protein, and AG-COS7 cells that stably express both ACE2 and green fluorescence protein, respectively. When SR-COS7 cells or R-COS7 cells were cocultured with AG-COS7 cells, syncytia with yellow fluorescence were conveniently observed after 12 h in SR-COS7 cells plus AG-COS7 cells, but not in R-COS7 cells plus AG-COS7 cells. The cell-to-cell fusion efficiency was simply determined for quantitative analysis based on the number of syncytium detected by flow cytometry. Such new cell-to-cell fusion model was further assessed by the potent HR2 peptide inhibitor, which led to the obvious decrease of the cell-to-cell fusion efficiency. The successful fusion and inhibition of cell-based binding assay shows that it can be well used for the study of SARS-CoV entry and inhibition.

IUBMB *Life*, 58: 480–486, 2006

Keywords SARS-CoV; S protein; ACE2; cell-to-cell fusion assay; fusion inhibition.

INTRODUCTION

The SARS coronavirus (SARS-CoV) is the agent of severe acute respiratory syndrome, whose emergence resulted in serious epidemic in 2002–2003. In order to prevent and treat the potentially deadly viral infection, researchers around the world pooled their scientific resources in an unprecedented manner to study characteristics of SARS-CoV (1, 2), elucidate the viral fusion mechanism (3–5), and develop potential

vaccines and drugs (6–8) in recent years. However, there are still large gaps in the knowledge about the pathogenesis of the virus.

Nowadays, the study of SARS-CoV infection mainly focuses on elucidating the fusion mechanism and developing the fusion inhibitors. During the viral entry process, spike (S) protein on the virion surface is involved in receptor binding and membrane fusion between viral and cellular membranes. Although S protein of SARS-CoV does not appear to be cleaved into two segments, it still contains S1 and S2 functional domains. The S1 bears the receptor attachment site of angiotensin-converting enzyme 2 (ACE2) (9, 10), and the S2 has the fusion activity through forming a trimer-of-hairpins structure (11, 12). The special structure and function of S protein are applied to screen potential fusion inhibitors through inhibiting formation of trimer-of-hairpin (4, 6). In the study of viral entry and inhibition, viral infectivity assay is usually necessary. For the high contagious viruses, especially for SARS-CoV that was strictly used in routine research in some countries and regions, cell-to-cell fusion model is needed to reduce the usage of live virus. Usually, the cell-to-cell fusion assay can reconstitute interaction between viral glycoprotein and its cellular receptor. Now such cell-to-cell fusion model is widely used in the field of HIV-1 for the interaction between gp120 and gp41 (13), structure-function relationship of gp41, steric accessibility of the HIV-gp41 N-trimer region (14), and screen of potential inhibitors (15, 16). Considering seriously restrictive usage of SARS-CoV and benefits of cell fusion assay, a convenient and safe cell-to-cell fusion assay is greatly needed for the study of SARS-CoV entry inhibitors.

Nowadays there are two main cell-to-cell fusion models simulating SARS-CoV infection. One is cell-to-cell fusion between the Vero E6 cells, a SARS-CoV receptor (ACE2) expressing cell-line, and the transfected cells expressing EGFP-fused S protein (17), and the other is luciferase activity-based cell-to-cell fusion assay (18, 19). These cell-to-cell fusion

Received 26 April 2006; accepted 22 May 2006

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models were used for studying viral entry and inhibition, but their cell-binding approaches were not visualized enough. In this work, we tried to develop a safe and convenient cell-to-cell fusion assay for the study of SARS-CoV entry and inhibition. One pool of COS7 cells stably expresses both SARS-CoV S protein and red fluorescence protein, and another pool of COS7 cells stably expresses both ACE2 and green fluorescence protein, respectively. The cell-to-cell fusion process is conveniently monitored according to the formation of syncytia with yellow fluorescence, and the fusion efficiency is simply detected by flow cytometry. Therefore, such fusion assay can be well used for the study of SARS-CoV fusion mechanism and the identification of potential inhibitors.

MATERIAL AND METHODS

Gene Constructions

The SARS-CoV S protein gene was amplified by PCR from SARS-CoV WHU strain (GenBank Accession No. AY394850), which was isolated from a SARS patient. The fragment was cloned into pCMV-Tag2B vector (Clontech, USA) to generate the plasmid pS. The plasmid ACE2-pCDNA3.1(-) was kindly provided by Dr GengFu Xiao (State Key Laboratory of Virology, China). The ACE2 gene was amplified by PCR from the plasmid ACE2-pCDNA3.1(-) and cloned into pIRES2-EGFP vector (Clontech, USA) to generate pAG. The pIRES2-EGFP vector contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) and can be used to express EGFP alone or obtain stably transfected cell lines without time-consuming clonal selection. In this paper, the second heptad repeat region (HR2) region of SARS-CoV S protein covers amino acids 1149-1186 of S protein. The DNA fragment corresponding to the HR2 with 5 additional amino acid residues DDDDK (Enterokinase cleavage site) at the N-terminal was constructed by overlapping PCR and cloned into the *Bam*HI-*Xho*I restriction enzyme sites of the GST fusion expression vector pGEX-6p-1 (Pharmacia, Sweden). DNA sequencing confirmed that these recombinant vectors were constructed successfully.

Expression and Purification of Recombined Proteins

The N-terminal GST fused peptide, GST-HR2, was expressed in *E. coli* strain BL21 (DE3) and purified with glutathione-Sepharose 4B (Pharmacia, Sweden) according to the manufacturer's instructions with some minor modifications. Briefly, cells were grown in Luria Broth (LB) containing 100 µg/ml ampicillin at 37°C. When the culture density (OD₆₀₀) reached 0.6, the culture was induced with 0.2 mM Isopropyl-β-D-thiogalactoside (IPTG) and grown for an additional 4 h at 28°C. The harvested culture was centrifuged and the bacterial cell pellet was re-suspended in lysis Buffer (1% Triton X-100, PBS, pH 7.3) and homogenized by sonication. The lysate was subsequently clarified by

centrifugation at 12,000 rpm for 15 min at 4°C. Then the supernatant was loaded onto a glutathione-Sepharose 4B column. The protein-loaded column was then washed with PBS. After that, the GST-fusion protein, GST-HR2, was eluted with 10 mM reduced glutathione. For the GST-removed protein HR2, the enterokinase was added into the resin to cleave the GST, and the HR2 was eluted. GST-HR2 and HR2 were concentrated by freeze-drying and were desalted with molecular sieve. Proteins were analyzed on 10% Tris-tricine SDS-PAGE or 12% SDS-PAGE.

Transfection and Stable Selection of Cell Lines

COS7 cell line was obtained from China Center of Typical Culture Collection (CCTCC). To establish the SARS-CoV S protein or ACE2 expressing cell lines, approximately 50% density of COS7 cells on 6-well plates, were transfected by using SofastTM (Sunma, China) with pS plasmid encoding the SARS-CoV S protein (S-COS7) or pAG plasmid encoding the ACE2 (AG-COS7). Transfectants were selected by the addition of 800 µg/ml G418 (Merck, USA) in DMEM. For 2 weeks of selection, G418-resistant colonies were isolated and expanded in tissue culture plates. Then the plasmid of pDsRed2-ER vector (Clontech, USA) expressing red fluorescence protein in endoplasmic reticulum (ER), was transfected into SARS-CoV S protein expressing cell line. The transfectants with red fluorescence (SR-COS7) were picked out under fluorescent microscope and cultivated in fresh complete medium containing 800 µg/ml G418. Acting as control of the cell-based binding assay, a stably expressing COS7 cell line (R-COS7) was generated by introducing the plasmid pDsRed2-ER into the pCMV-Tag2B stable transfectants.

Western Blot Analysis

Expression of SARS-CoV S protein was further confirmed by western blotting. Cell lysates were prepared by solubilizing cells at 2×10^7 cells/ml in the lysis buffer containing 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 1% triton X-100 and 1 mM phenyl-methylsulfonyl fluoride (PMSF, Sigma), followed by centrifugation at 12,000 rpm for 10 min. Supernatants were collected, mixed with an equal volume of reducing SDS-PAGE 2 × sample buffer, aliquoted and stored at -80°C. Lysates of the transfectants were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, Bedford, MA) by standard methods. After blocked with PBST containing 5% non-fat milk for 1 h, the membrane was incubated with sera (1:200, from rabbits immunized with GST-HR2 of the S protein) in TTBS for 3 h at 37°C, washed three times with TTBS. The HRP-labeled goat anti-rabbit IgG was used as the second antibody and then was incubated for 1 h at room temperature. The color reaction was revealed by incubation in 0.1 M Tris-HCl pH 6.8, containing 0.5 mg/ml diaminobenzidine (DAB, Sigma) and 0.01% H₂O₂.

RNA Preparation and RT-PCR Analysis

The ACE2 expression of transfectants was demonstrated by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from transfected COS7 cells using Trizol[®] LS Reagent (Gibco-BRL). Potentially contaminating DNA was removed by treating with RNase-free DNase I (Promega, USA). First strand cDNA was prepared by reverse transcription using reverse transcriptase (Gibco-BRL) according to the manufacturer's instructions. The expression of ACE2 was analyzed with the following primers: forward 5'-GCCACCATGTCAAGCTCTTCCTGGCTC-3'; reverse 5'-GTGCACAAGCTTAAG CGGGCGCCACCTG-3'.

Cell-to-cell Fusion and Inhibition Assay

The cell-to-cell fusion assay was used for assessing the ability of the S protein to mediate receptor-dependent cell-to-cell fusion. The effector cells are stable transfected COS7 cells expressing SARS-CoV S protein with red fluorescence (SR-COS7), and the target cells are stable transfected COS7 cells expressing ACE2 with green fluorescence (AG-COS7). All SR-COS7 cells and AG-COS7s cell were firstly trypsinized, then re-suspended in DMEM-10% FBS (pH 7.5) and mixed with 1:1 ratio. After these cells were cocultured for 12 h at 37°C, multinucleated syncytia with yellow fluorescence could be observed under fluorescence microscope. The efficiency of viral S protein mediated-cell fusion was determined by flow cytometry, which can calculate easily the number of the syncytium emerging both red fluorescence and green fluorescence. For the sample preparation to be detected by flow cytometry, the cells were trypsinized and centrifugated at 3,300 rpm for 8 min, followed by wash with PBS 2 times. Then the cells at 2×10^7 cells/ml were solubilized in the buffer containing 30% PBS and 70% ethanol and could be detected by flow cytometer with two record filters. In this inhibition analysis, HR2 peptide was added into DMEM after the cells were trypsinized, re-suspended and mixed at 1:1 ratio. After the mixture of cells coculture at 37°C for 12 h, the number of syncytium showing bicolor fluorescence was monitored by flow cytometry.

RESULTS

Soluble Expression and Purification of GST-HR2 and HR2

The whole HR region of SARS-CoV S protein plays an important role in the process of virus infection. Synthetic peptide from the second HR region is a potent inhibitor of virus entry that could inhibit SARS-CoV fusion (4, 7, 8). In this study, HR2 peptide was selected for recombinant expression. We found that GST fused protein of HR2 was expressed in *E. coli* at high level as soluble protein in appropriate temperature and induced time. Simultaneously, both GST fused protein of HR2 and GST-removed peptide of HR2 were also soluble in PBS. The results of protein analysis on Tris-tricine

SDS-PAGE or SDS-PAGE demonstrated the purification approach of GST-HR2 and HR2 were efficient. As shown in Fig. 1, only single band was presented in the samples of recombinant GST-HR2 or HR2, and the molecular weights of GST-HR2 band and HR2 band were respectively estimated approximately as 30 KDa and 4 KDa to accord with the true features.

Expression of SARS-CoV S Protein

In this work, SR-COS7 cells were transfected successively with pS and pDsRed2-ER and expressed both SARS-CoV S and red fluorescence proteins. In order to confirm the correct expression of SARS-CoV S protein, western blotting was performed utilizing rabbit polyclonal antibody obtained by immunization of rabbit with GST-HR2. The validity of the polyclonal antibody specific for GST-HR2 was identified by western blot analysis (Fig. 2, lane 4). As shown in Fig. 2, the polyclonal antibody can specifically recognize a band of ~180 KDa (lane 2), which is consistent with the molecular weight of SARS-CoV S protein. However, there is no band in the corresponding position for the vector control-transfected cells (lane 3). These results demonstrated that SARS-CoV S protein was successfully expressed in SR-COS7 cells.

SARS-CoV Receptor ACE2 Expression

We constructed a recombinant expression vector pAG by using IRES-mediated construction, which was expected to express both ACE2 and EGFP, respectively. The strategy was convenient to monitor the expression of ACE2 by fluorescence microscope. Further confirmation of ACE2 expression at

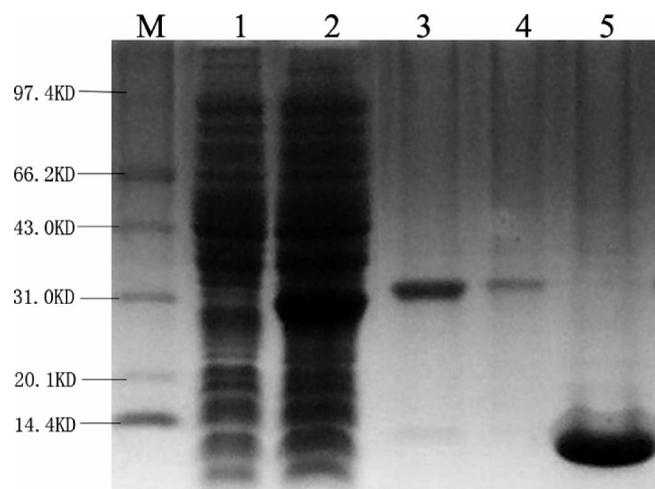


Figure 1. Protein analysis on Tris-tricine SDS-PAGE. Lane M: Marker proteins. Lane 1: The expression of GST-HR2 in the no-induced bacterium. Lane 2: The expression of GST-HR2 in the induced bacterium. Lane 3–4: The purified GST-HR2 emerging a band about 30 KDa. Lane 5: The purified HR2 emerging a band about 4 KDa.

the mRNA level was carried out by RT-PCR with primers specific for the gene in this work. As shown in Fig. 3, a 2400 bp DNA fragment specific for ACE2 was detected in the AG-COS7 cells-derived samples, while no 2400 bp DNA

fragment specific for ACE2 were observed in the samples derived from pIRES2-EGFP transfectants (G-COS7). When the RNA of Vero E6 was conducted as templates of RT-PCR, a 2400 bp DNA fragment specific for ACE2 also could be detected. This indicated that Vero E6 was ACE2-positive cell and the ACE2 could be express in AG-COS7 cells.

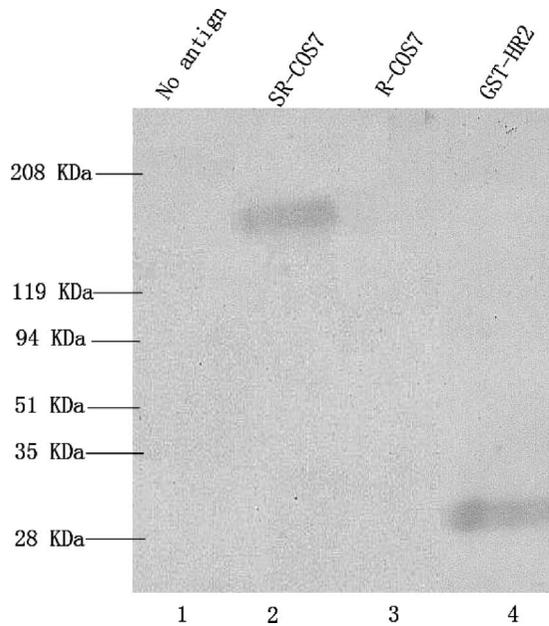


Figure 2. Western blot analysis of the expression of SARS-CoV S protein. Lane 1: No antigen was added in the process for negative control. Lane 2: Western blot analysis of cell lysates from SR-COS7. Lane 3: Western blot analysis of cell lysates from R-COS7. Lane 4: Western blot analysis of GST-HR2 to detect the quality of the polyclonal antiserum.

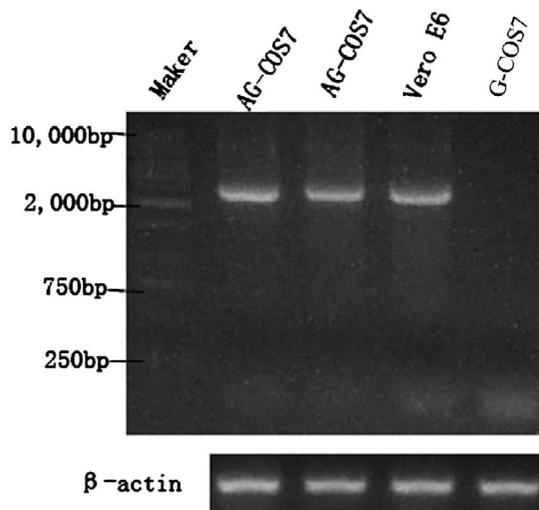


Figure 3. RT-PCR analysis of the expression of ACE2 in stable transfected AG-COS7 cells. Templates: Lane 1-2, The RNA of AG-COS7. Lane 3, The RNA of Vero E6. Lane 4, The RNA of the stable pIRES-EGFP control transfectants.

Cell-to-cell Fusion between SR-COS7 Cells and AG-COS7 Cells

Interaction between SARS-CoV S protein and receptor ACE2 mediating membrane fusion can be performed through cell-to-cell fusion. In this study, we demonstrated the binding activity between the S protein expressing cells and ACE2 expressing cells. When the S protein expressing cells with red fluorescence (SR-COS7, Fig. 4a) were mixed with cells expressing receptor ACE2 with green fluorescence (AG-COS7, Fig. 4b) at 1:1 ratio and incubated for 12 h, syncytia with yellow fluorescence were observed by fluorescence microscope (Fig. 4c), but no syncytium was detected in R-COS7 plus AG-COS7 cells (Fig. 4d). In this cell-to-cell fusion experiment, the effect of different pH values ranging from pH 5.0 to pH 8.5 on cell-to-cell fusion was investigated, and it was found that neutral pH value is more beneficial to cell-to-cell fusion. In addition, we also found that the percent of syncytium was much higher than that in other conditions when the density of mixed cells is about 30%, and the incubated time is 12–18 h. At the optimized conditions, the overall percent of syncytia fused between SR-COS7 and AG-COS7 cells was about 10% (Fig. 5a).

Inhibition Analysis of Cell-to-cell Fusion

In order to inhibit SARS-CoV entry into the host cell, series of peptides were designed and their inhibitory abilities were tested (4, 7, 8). Among these peptides, HR2 peptide had the best inhibition activity at nM level (8). In this work, recombinant HR2 peptide was expressed successfully (Fig. 1), and it was used for assessing whether our cell-to-cell fusion process can be blocked. For the test of fusion inhibition, SR-COS7 and AG-COS7 cells were trypsinized respectively and mixed at the ratio of 1:1 in 6-well plates, then HR2 peptides was added. After mixture of cells was incubated for 12 h at 37°C, we could see much less number of syncytia under fluorescence microscope than the number in the absent of HR2. By using flow cytometry to detect the efficiency of the inhibition according to the number of bicolor fluorescence cell, the inhibitory efficiency was about 60% in the presence of 50 μ M HR2 peptide (Fig. 5b). All inhibition experiments showed coincident results that the cell-to-cell fusion could be effectively inhibited at μ M range by HR2 peptide.

DISCUSSION

SARS-CoV is so highly infectious that its usage has been strictly restricted for most laboratories, which has been

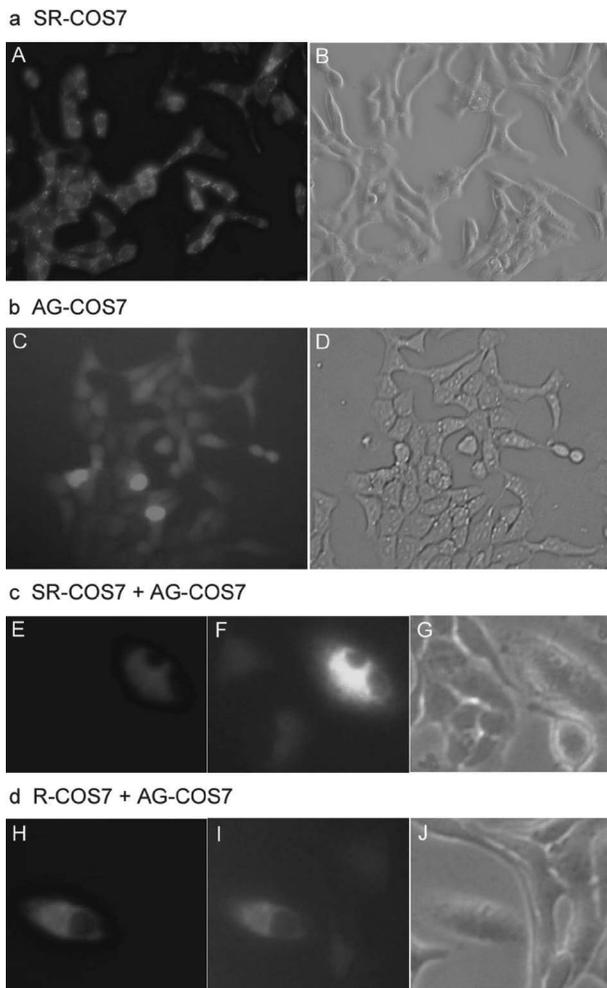


Figure 4. Cell-to-cell fusion assay. (a) SR-COS7 cells under fluorescence microscope (A) and under bright field (B). (b) AG-COS7 cells under fluorescence microscope (C) and under bright field (D). (c) Cell-to-cell fusion between SR-COS7 cells and AG-COS7 under fluorescence microscope with different filters. (E: Under fluorescence microscope with green excitation light. F: Under fluorescence microscope with blue excitation light. G: Under bright field for control.) (d) Coculture between R-COS7 cells and AG-COS7 cells. (H: Under fluorescence microscope with green excitation light. I: Under fluorescence microscope with blue excitation light. J: Under bright field for control.)

seriously affecting series of fundamental research on viral entry and inhibition. As for HIV-1, both cell-to-cell fusion and viral infection assays are widely used in the field of fusion mechanism and screening of potential inhibitors (14, 16, 20). Considering seriously restrictive usage of SARS-CoV strain and benefits of cell-to-cell fusion assay, we tried to develop a convenient cell-to-cell fusion assay in this work. The fusion process was conducted between SR-COS7 cells with red fluorescence protein and AG-COS7 cells with green

fluorescence protein, which successfully confirmed the function of viral S protein mediating membrane fusion between virus and host cell. In this work, flow cytometry was applied to determine cell-to-cell fusion efficiency based on its characteristic that can easily count the number of SR-COS7 cell, AG-COS7 cell and syncytium according to the fluorescence in cell, respectively. Compared with cell-to-cell fusion assay of SARS-CoV between Vero E6 cells (a SARS-CoV receptor ACE2 expressing cell line) and transfected cells expressing EGFP-tagged S protein (17), the obvious advantage was that this cell-to-cell fusion process was considerably convenient to be monitored according to the yellow fluorescence of syncytia. Simultaneously, lack of quantitative analysis for cell-to-cell fusion efficiency (17) was also overcome in our cell-to-cell fusion assay. In this work, quantitative analysis of cell-to-cell fusion was simply realized by counting the number of syncytium with flow cytometry. Perhaps, the actual cell-to-cell fusion efficiency might be higher than the results from flow cytometry because fused syncytia were fractured more easily than the single cell during the course of trypsinization. The quantitative analysis of cell-to-cell fusion based on the luciferase activity was used for functional research of S protein (19, 21) and potential inhibitor identification (4, 7, 18). Compared with this cell-to-cell fusion assay, the luciferase activity-based cell-to-cell fusion assay was not visualized enough, and it was difficult to directly determine how many cells were fused in the cell-to-cell fusion assay. In addition, the successful inhibition of cell-to-cell fusion blocked by HR2 peptide not only showed that there were interaction between S protein and ACE2 but also confirmed that the mechanism of trimer-of-hairpins structure (11, 12) was adopted during this cell-to-cell fusion process.

Different pH values are important for different viral entry process. For example, HIV-1 infects through a pH-independent cell-to-cell fusion and entry process (22). In contrast, influenza virus utilizes a pH-dependent endocytotic pathway. As for SARS-CoV entry, the effect of pH seems unclear until now. The pH-dependent fusion mediated by the SARS-CoV S protein was ever found, and addition of ammonium chloride was helpful for SARS-CoV entry (18, 22). However, the neutral pH was then found necessary for cell-to-cell fusion (3). In our work, the effect of different pH values ranging from pH 5.0 to pH 8.5 on cell-to-cell fusion was investigated, and it was found that neutral pH is much beneficial to cell-to-cell fusion. Such results further confirmed that the interaction between S protein expressing cells and ACE2 expressing cells could well induce cell membrane fusion at neutral pH.

In addition, the polycistronic vector was constructed by using IRES-mediated methods in this work according to the previous studies (23, 24). One of the advantages of the IRES-mediated polycistronic expression vector is to eliminate redundant control regions such as internal promoters and terminators, and thus reduce plasmid size and facilitate efficient transfection. Moreover, functional studies showed

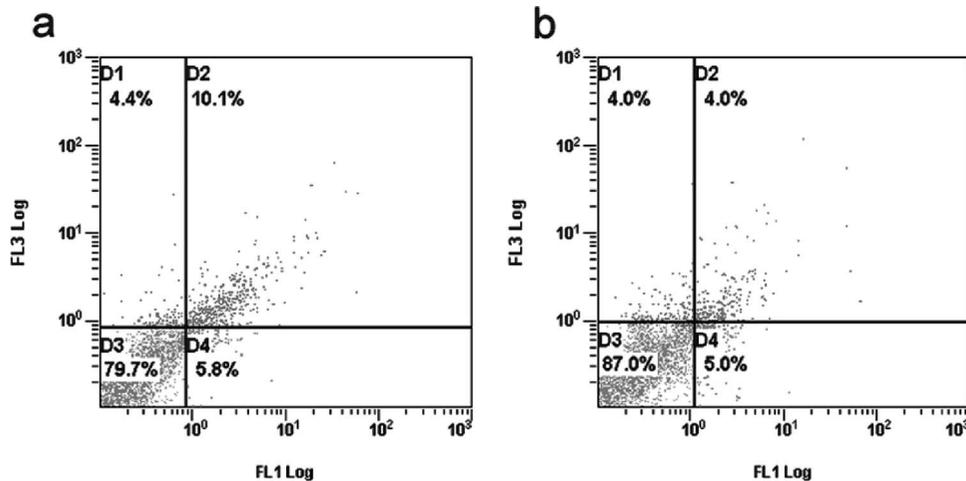


Figure 5. Inhibition assay of cell-to-cell fusion by flow cytometry. (a) Cell-to-cell fusion assay in the absence of HR2 peptide. (b) Cell-to-cell fusion assay in the presence of HR2 peptide.

that simultaneous expression of SARS-CoV receptor ACE2 and fluorescence protein could provide more creditable results to detect the process of SARS-CoV entry and inhibition than that of EGFP-fused ACE2.

Although a convenient cell-to-cell fusion assay was developed for SARS-CoV entry and inhibition, there was one common shortage for cell-to-cell fusion assays, which was the low fusion cell efficiency. Some studies also reported that there were average 10 syncytia per well in 96-well plates (3). During our cell-to-cell fusion assay, although the fusion efficiency increased, about 10% syncytia detected by flow cytometry was not enough. Therefore, how to further improve the cell-to-cell fusion efficiency is our future focus. At present, the improved cell-to-cell fusion assay is much useful to study SARS-CoV fusion mechanism and screen potential inhibitors.

ACKNOWLEDGEMENTS

This work was supported by China 973 basic research program grant 2003CB514120. We thank Dr ChangJiang Weng for his helpful discussion on the manuscript.

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