Evaluation of a Novel Vesicular Stomatitis Virus Pseudotype-Based Assay for Detection of Neutralizing Antibody Responses to SARS-CoV

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Severe acute respiratory syndrome (SARS)-coronavirus (SARS-CoV) is the causative agent of SARS. The S protein of SARS-CoV is a major target for neutralizing antibodies (Nabs) in infected patients. We developed a neutralization assay using a recombinant vesicular stomatitis virus (VSV) bearing SARS-CoV-S protein (VSV-SARS-St19). A total of 56 serum samples collected from 22 healthcare workers in the Hanoi French Hospital during the SARS epidemic in 2003 were evaluated and compared to the conventional neutralizing assay using infectious SARS-CoV. The results of the neutralization assay using VSV-SARS-St19 pseudotype showed good correlations with those using infectious SARS-CoV. The newly developed neutralization assay was more sensitive to low antibody titers in serum samples. Thus, the VSV-SARS-St19 is a useful tool for detecting Nabs against SARS-CoV. J. Med. Virol. 78:1509-1512, 2006.

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KEY WORDS: SARS coronavirus; VSV; pseudotype; neutralization

INTRODUCTION

Severe acute respiratory syndrome (SARS) is a recently emerging life-threatening respiratory disease that has created international concern because of its novelty, communicability, and rapid spread leading to a worldwide outbreak in 2003 [WHO, 2003]. The development of early, rapid, and reliable diagnostic assay systems is a high priority for early vigilance to prevent the spread of the disease. Identification of SARScoronavirus (SARS-CoV) as the causative agent of SARS has led to the development of genome-based and serological assays for diagnosis of viral infection [Hong et al., 2004; Hourfar et al., 2004; Saijo et al., 2005]. Detection of virus neutralizing antibody (Nab) is

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important to assess immune responses in SARS-CoVinfected patients. However, conventional virus neutralization assays require handling of infectious, replication-competent SARS-CoV, which raises a number of important safety concerns. Furthermore, it takes at least 2 days to obtain results, because detectable levels of virus replication are required.

Pseudotyped viruses provide a safe tool for virological studies because of their inability to produce infectious progeny virus [Takada et al., 1997]. A quantitative assay with pseudotyped virus infection could facilitate research on virus entry, cell tropism, and virus neutralization. Entry of SARS-CoV into susceptible cells is mediated by binding of the viral S protein to the receptor molecule, angiotensin I-converting enzyme II (ACE2) [Li et al., 2003]. Recently, we reported that recombinant vesicular stomatitis virus (VSV) in which the G gene has been replaced by the green fluorescent protein (GFP) gene, could be pseudotyped with S protein of SARS-CoV [Fukushi et al., 2005]. Infection with SARS-CoV-S protein-bearing VSV pseudotype (VSV-SARS-St19) is mediated by S protein in an ACE2-dependent manner [Fukushi et al., 2005]. As the SARS-CoV-S protein is a major target for Nabs [Hofmann et al., 2004], it is expected that Nabs in human serum samples also neutralize VSV-SARS-St19. In the present study, a novel Nab assay using VSV-SARS-St19 was developed and its performance was compared with that of a conventional Nab assay using infectious SARS-CoV.

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MATERIALS AND METHODS

Serum Samples

Fifty-six serum samples collected from 22 healthcare workers in the Hanoi French Hospital, Ho Chi Min City, during a SARS outbreak from February to April 2003 [Saijo et al., 2005] were used in this study. The sera were used for serological analyses after heat-inactivation at 56° C for 30 min.

SARS-CoV

SARS-CoV (HKU39849) used in the present study was kindly supplied by Dr. J.S. Malik Peiris, Department of Microbiology, University of Hong Kong. The virus was propagated on Vero E6 cells and the infectious titer (plaque forming U/ml) was determined on Vero E6 cells.

VSV-SARS-St19 Pseudotype

Generation of VSV-SARS-St19 was performed as described previously [Fukushi et al., 2005]. Briefly, at 24 hr after transfection of 293T cells with pKS-SARS-St19, an expression plasmid encoding a C-terminaltruncated version of the SARS-CoV-S protein, the cells were infected with VSV Δ G* (kindly provided by Dr. M.A. Whitt, GTx, Inc.). After absorption for 1 hr, the inoculum was replaced with culture medium and cultured for 24 hr at 37°C in a CO₂ incubator. The culture supernatants were then collected, filtered through a 0.22-µm-pore size filter, and stored at -80° C until use. The titer (infectious units, IU) of pseudotype viruses, which means the number of GFP-positive cells, was determined by end-point dilution using Vero E6 cells.

Neutralizing Assay

As pseudotype VSVs do not produce infectious progeny virus, pseudotype-based neutralization requires higher amounts of pseudotype virus (3,000 IU) than that of SARS-CoV (100 plaque forming units, PFU) in the conventional neutralization assay. The 3,000 IU of VSV-SARS-St19 was the optimum amount for the pseudotype-based neutralization assay. The serum samples were diluted twofold from 1:80 to 1:5,120 with Dulbecco's Modified Eagle's medium (DMEM), containing 5% fetal bovine serum (FBS) and 3,000 IU of VSV-SARS-St19. The mixture was incubated for 1 hr at 37°C for neutralization. After incubation, the mixture was inoculated onto Vero E6 cells seeded on 96-well plates. The infectivity of VSV-SARS-St19 was determined by counting of the number of GFP-positive cells according to the methods described previously [Fukushi et al., 2005]. The Nab titer was defined as the reciprocal of the highest dilution at which more than 50% inhibition of infectivity was observed. The conventional neutralization assay using infectious SARS-CoV (isolate HKU39849, kindly supplied by Dr. J.S. Malik Peiris) was performed as described by Saijo et al. [2005]. Briefly,

the heat-inactivated serum samples were diluted twofold with Eagle's minimum essential medium (MEM) containing 2% FBS from 1:10 to 1:320. Each test sample (60 μ l by volume) was then mixed with the same volume of SARS-CoV at an infectious dose of 100 PFU and the mixture was incubated for 1 hr at 37°C for neutralization. After incubation, the mixtures were tested for neutralization by cytopathic effect (CPE) inhibition assay using Vero E6 cells. The cutoff value was set at a serum dilution of 1:20. The Nab titer was defined as a reciprocal of the highest dilution at which no CPE was observed.

RESULTS AND DISCUSSION

Recently, we reported that a rabbit antibody raised against purified, inactivated SARS-CoV neutralized VSV-SARS-St19 infection of Vero E6 cells [Fukushi et al., 2005]. To examine whether the Nabs induced in SARS-CoV-infected patients neutralize VSV-SARS-St19 infection, serum samples collected from healthcare workers in the Hanoi French Hospital during the SARS epidemic in 2003 were used. Although clinical information on these subjects was not available, the sera were shown to be sero-converted by conventional SARS-CoV neutralization assay or SARS-CoV-recombinant NP (rNP)-based ELISA [Saijo et al., 2005]. As some SARS-CoV antibody-negative control human sera showed nonspecific anti-pseudotype activity up to a serum dilution of 1:40 (data not shown), samples were tested at dilutions of 1:80 or more, with this value set as the cutoff. VSV-SARS-St19 was preincubated with serum samples serially diluted from 1:80 to 1:5,120. Then, the preincubated virus was inoculated onto Vero E6 cells and infectious foci were counted. Figure 1 shows dynamic cumulative percentages of pseudotype infectivity using serum samples collected from three subjects (S1, S2, and S3). Samples were considered Nab-positive when VSV-SARS-St19 infection was inhibited by 50% or greater as compared to serum-negative control. There were no Nabs detected in the serum samples collected at earlier stages (i.e., serum samples collected on March 17 from S1 and S3 and serum sample from S2 collected on March 27), as the infectivity of the pseudotype in the presence of diluted serum samples ranged from 70.1 to 101.7% of the serum negative control (data not shown). In contrast, the serum samples collected from these three subjects at later stages (March 29) showed a reduction in the number of foci by more than 50% (Fig. 1). The results indicated that these serum samples contained Nabs to VSV-SARS-St19. The Nab titers were defined as the reciprocal of the highest dilution at which more than 50% inhibition of infectivity was observed. Serum samples collected on March 29 from S1, S2, and S3 were shown to have Nabs with titers of 1,280; 320; and 320, respectively. Nab titers using SARS-CoV on conventional neutralization assay for S1, S2, and S3 were >640, 80, and 160, respectively (data not shown). The results of neutralization assay using VSV-SARS-St19 were in good agreement with those of neutraliza-



Fig. 1. Neutralization assay using VSV-SARS-St19 pseudotype. A: Inhibition of VSV-SARS-St19 infection by serum samples. Prior to infection of Vero E6 cells, VSV-SARS-St19 pseudotypes were preincubated with two serum samples collected from each of three subjects (S1, S2, and S3) at the indicated dilutions. Serum samples were collected at March 17 and 29 for the subjects S1 and S3, whereas serum samples were collected at March 27 and 29 for the subject S2. The infectivity of the pseudotype, determined by counting GFP-positive cells, in the absence of serum was considered 100%. The infectivity of the pseudotype in the presence of diluted serum samples collected at later stages (March 29, 2003) was shown.

tion assay using SARS-CoV and Nab titers obtained with VSV-based neutralization assay were two- to fourfold higher than those obtained with conventional neutralization assay.

To validate the VSV-SARS-St19-based neutralization assay, an additional neutralization assay was carried out on a subset of 53 serum samples collected from healthcare workers in the Hanoi French Hospital. The titers of Nabs determined using VSV-SARS-St19 were compared with those using a conventional neutralization assay with infectious SARS-CoV. Thirty-three serum samples were shown to be positive by either neutralization assay. As shown in Figure 2, there was a significant positive correlation (correlation coefficient = 0.77) between the Nab titers determined by VSV-SARS-St19 and SARS-CoV. Of the 36 serum samples positive by VSV-SARS-St19 neutralization assay, 3 were negative by the conventional neutralization assay (Table I). The possibility of false-positive results of these three samples in VSV-SARS-St19-based assay cannot be excluded. However, it seems likely that these three sera had extremely low titers of Nabs to SARS-CoV, as sera collected from the same subjects 9 or 14 days later had high Nabs titers (from 320 to 640) to VSV-SARS-St19 (data not shown). Furthermore, among these three serum samples, one was positive by rNP-based ELISA with an OD_{405} value of 0.568 at 1:100 dilution (data not shown), indicating that the serum contained antibodies to SARS-CoV. Taken together, these results suggest that the VSV-SARS-St19-based neutralization assay was more sensitive than the conventional neutralization assay using SARS-CoV. This assumption was supported by the observation that the Nab titers measured using



Fig. 2. Correlation between neutralizing antibody titers measured using VSV-SARS-St19 pseudotype and those measured using SARS-CoV. The correlation coefficient was 0.77. Nab titers (log_{10}) values with SARS-CoV neutralization assay plotted between 1 and 3 on the y-axis scale whereas those with VSV-based assay plotted between 1 and 4 on the x-axis scale.

VSV-SARS-St19 were higher than those measured by the conventional neutralization assay (Figs. 1 and 2). Neutralization data obtained with 56 serum samples, with neutralization assay using SARS-CoV as a reference method, showed that the sensitivity and specificity of VSV-SARS-St19-based neutralization assay were 97 and 86%, respectively (Table I). Neutralization assay is the gold standard in testing for antibodies to SARS-CoV because of its specificity and sensitivity. As comparative neutralization data indicated a good correlation with conventional neutralization assay using SARS-CoV, VSV-SARS-St19-based neutralization assay is a reliable serological test for SARS-CoV infection.

The pseudotype-based neutralization assay does not require handling of infectious SARS-CoV. This safety concern has led several laboratories to utilization of replication-incompetent retrovirus-based SARS-CoV-S pseudotype for assessing Nabs to SARS-CoV [Nie et al., 2004; Temperton et al., 2005]. The retrovirus pseudotype-based neutralization assay is shown to be both sensitive and specific for conventional neutralization assay [Nie et al., 2004; Temperton et al., 2005]. However, time required to determine the virus infectivity in the

TABLE I. Comparison of the Results of the Neutralization Assay Using VSV-SARS-St19 Pseudotype With Those Using SARS-CoV

SARS-CoV	VSV-SARS-S pseudotype		
	Positive	Negative	Total
Positive Negative Total	33 3ª 36	$\begin{array}{c}1\\19\\20\end{array}$	$34 \\ 22 \\ 56$

^aOne serum sample was positive in the rNP-based ELISA.

retrovirus system is 48 hr, 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. In contrast, use of the VSV Δ G* system has the advantage of rapid detection of pseudotype infection [Ogino et al., 2003]. Recently, we reported that upon infection of Vero E6 cells by VSV-SARS-St19 pseudotype, infected cells can be detected at 7 hr post-infection due to rapid expression of GFP in the VSV Δ G* system [Fukushi et al., 2005]. Furthermore, quantitative analysis of VSV-SARS-St19 infection can be performed easily by counting the number of GFP-positive cells using ImageJ software (http://rsb.info.nih.gov/ij/). Thus, this novel Nab assay system allows the measurement of SARS-CoV-specific Nabs within 1 day.

In the present study, we established a rapid and safe SARS-CoV-neutralization assay using VSV-SARS-St19 pseudotype. The results obtained using this system showed a good correlation with those obtained using the conventional neutralization assay with SARS-CoV. Furthermore, the novel Nab assay appeared to be more sensitive to low antibody titers in serum samples. Thus, VSV-SARS-St19 provides a useful tool for detecting Nabs against SARS-CoV.

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