Exogenous ACE2 Expression Allows Refractory Cell Lines To Support Severe Acute Respiratory Syndrome Coronavirus Replication

Eric C. Mossel,1* Cheng Huang,1 Krishna Narayanan,1 Shinji Makino,1 Robert B. Tesh,2 and C. J. Peters1,2

Department of Microbiology and Immunology1 and Department of Pathology,2 University of Texas Medical Branch, Galveston, Texas

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Of 30 cell lines and primary cells examined, productive severe acute respiratory syndrome coronavirus (Urbani strain) (SARS-CoV) infection after low-multiplicity inoculation was detected in only six: three African green monkey kidney epithelial cell lines (Vero, Vero E6, and MA104), a human colon epithelial line (CaCo-2), a porcine kidney epithelial line (PK(15)), and mink lung epithelial cells (Mv 1 Lu). SARS-CoV produced a lytic infection in Vero, Vero E6, and MA104 cells, but there was no visible cytopathic effect in Caco-2, Mv 1 Lu, or PK(15) cells. Multistep growth kinetics were identical in Vero E6 and MA104 cells, with maximum titer reached 24 h postinoculation (hpi). Virus titer was maximal 96 hpi in CaCo-2 cells, and virus was continually produced from infected CaCo-2 cells for at least 6 weeks after infection. CaCo-2 was the only human cell type of 13 tested that supported efficient SARS-CoV replication. Expression of the SARS-CoV receptor, angiotensin-converting enzyme 2 (ACE2), resulted in SARS-CoV replication in all refractory cell lines examined. Titers achieved were variable and dependent upon the method of ACE2 expression.

The family Coronaviridae consists of large enveloped positive-sense RNA viruses causing mild to severe enteric and respiratory diseases in humans and animals (15, 19). In 2003, a novel coronavirus was identified as the etiological agent of a newly described respiratory illness, severe acute respiratory syndrome (SARS) (8, 17, 25). During the initial 9-month outbreak, the virus spread around the world, infecting 8,096 people and resulting in over 774 deaths (http://www.who.int/csr/sars/country/table/2004_04_21/en/).

The emergence of SARS coronavirus (SARS-CoV) represents an exception to the coronavirus epidemiology paradigm. Coronaviruses are known to infect numerous species, including mice, cows, dogs, cats, pigs, birds, and humans. However, individual virus strains are remarkably species specific, usually causing limited disease outside their natural host (15, 19). SARS-CoV is proposed to be an animal coronavirus that causes severe disease in humans (26). The putative natural reservoir of SARS-CoV is the Himalayan or masked palm civet, Paguma larvata (order Carnivora, family Viverridae). Additional virologic and serologic evidence suggests the raccoon dog and Chinese ferret badger (order Carnivora, families Canidae and Mustelidae, respectively) may also be naturally infected with SARS-CoV (12). The extent of SARS-CoV distribution in nature is not known, nor is it known whether the civet represents the true natural host or an intermediate host between the natural reservoir and humans.

Laboratory infections with SARS-CoV have been reported in cynomolgous and rhesus macaques, African green monkeys, cats, ferrets, hamsters, and mice (10, 13, 22, 27, 30; K Subbarao, A. Roberts, L. Vogel, and B. R. Murphy, Abstr. 23rd Annu. Meet. Am. Soc. Virol., abstr. W54-2, 2004; J. M. McAuliffe, L. Vogel, M. E. St. Claire, B. Murphy, and K. Subbarao, Abstr. 23rd Annu. Meet. Am. Soc. Virol., abstr. W54-3, 2004). In vitro host range data have been gathered as a specific goal and as a by-product of other studies. Vero E6, Mv 1 Lu, CaCo-2, Huh-7, FRhMK, 293, and LoVo cells have been reported to support SARS-CoV replication in culture, while many more cell lines have been reported to be refractory to SARS-CoV infection (2, 4, 11, 17, 25; F. Weber and M. Spiegel, personal communication). Angiotensin-converting enzyme 2 (ACE2) was identified as a receptor for SARS-CoV in Vero E6 cells (21), and CD209L was recently identified as a potential alternate receptor (16). Other cellular determinants of virus replication are unknown.

This study confirms and extends the known SARS-CoV in vitro host range data, examining virus growth kinetics and determinants for virus replication in cell culture.

**Multiple cell lines support SARS-CoV replication.** To confirm and extend knowledge of the host range of SARS-CoV, we examined the permissiveness of 30 continuous and primary cell lines. The cell type, donor host, and source of the line are listed in Table 1. Cell monolayers were infected with SARS-CoV, Urbani strain, at low multiplicity (multiplicity of infection [MOI] = 0.001 to 0.005). Cells were examined daily for cytopathic effect (CPE). When CPE reached 75 to 90%, cell culture medium was harvested for virus titration by 50% tissue culture infective dose (TCID50) assay or plaque assay on Vero E6 cells. If no CPE was observed, culture supernatant was harvested 6 days postinfection (dpi).

Efficient SARS-CoV replication was observed in six different cell lines (Table 1). Three African green monkey kidney epithelial cell lines, Vero, Vero E6, and MA104, supported lytic replication. Three disparate cell lines, CaCo-2 (human colon epithelial), Mv 1 Lu (Aleutian mink lung epithelial), and PK(15) (porcine kidney epithelial), supported nonlytic repli-
for efficient SARS-CoV replication, raising the possibility of alternate receptor use in nonpermissive cells (20). The diverse orders represented by the permissive cell lines suggest that cellular determinants sufficient for virus replication are conserved across Mammalia but may not be present in all mammalian cell lines.

Multistep growth curves for SARS-CoV, Vero E6, MA104, and CaCo-2 cells were infected at low multiplicity (MOI = 0.001 to 0.005), and supernatant titers were determined at the indicated time points by plaque assay to determine relative multistep virus growth kinetics. SARS-CoV grew equally well and rapidly in both Vero E6 and MA104 cells. In both cell lines, maximal titers of about $2 \times 10^7$ PFU/ml were reached 36 hpi. Vero E6 monolayers showed evidence of CPE (i.e., cell rounding and detachment) at 36 hpi. CPE was maximal in Vero E6 cells by 48 hpi. Despite similar virus growth, CPE was delayed in MA104 cells by about 24 h. CPE appeared at 60 hpi. Vero E6 monolayers showed evidence of CPE (i.e., cell rounding and detachment) at 36 hpi. CPE was maximal in Vero E6 cells by 48 hpi. Despite similar virus growth, CPE was delayed in MA104 cells by about 24 h. CPE appeared at 60 hpi.

CaCo-2 cells can be persistently infected with SARS-CoV. A human colorectal carcinoma cell line, LoVo, was recently shown to support persistent replication of SARS-CoV (2). To determine whether CaCo-2 cells resolve SARS-CoV infection...
or are persistently infected, monolayers were infected at low multiplicity (MOI = 0.001 to 0.005) and at the indicated times after inoculation, and the entire volume of medium was removed for analysis and replaced with fresh medium. At 8 dpi, cultures were split 1:6. Monolayers were then allowed to grow and remain confluent for the remainder of the experiment. A maximum virus titer of approximately $10^7$ PFU/ml was reached 3 dpi (Fig. 2). By 8 dpi, virus titer decreased to $10^6$ PFU/ml. One to 5 weeks postinoculation, monolayers remained intact and titers were maintained at approximately $10^6$ PFU/ml. Six weeks after infection, monolayers began to show signs of disruption, likely contributing to the observed 10-fold decrease in virus titer.

Expression of human ACE2 renders refractory cell lines permissive for SARS-CoV replication. For some coronaviruses, it has been observed that host cell restriction is due exclusively to the lack of an appropriate receptor and that alteration of the coronavirus spike protein binding is sufficient to alter virus host range (5, 18, 28, 29, 31). Restriction of SARS-CoV replication in murine 3T3 cells was recently shown to be overcome by expression of human ACE2 (20). To determine whether the failure of refractory additional cell lines from multiple species to support SARS-CoV replication was due to the lack of an appropriate receptor, SARS-CoV replication was examined in cells transiently expressing human ACE2. ACE2 cDNA was cloned from CaCo-2 cells and inserted into the plasmid expression vectors pcDNA3.1 (Invitrogen, Carlsbad, Calif.), pCAGGS, or pCX4bsr (1). pcDNA3.1ACE2 and pCAGGSACE2 were transfected into cells with Lipofectamine Plus or Lipofectamine 2000 reagent (Invitrogen) per the manufacturer’s protocols. pCX4bsrACE2, a murine leukemia virus vector encoding ACE2, was cotransfected with a plasmid expressing amphotropic murine leukemia virus glycoproteins into BOSC23 cells to produce the ACE2-expressing pseudotyped retrovirus as described previously (3, 24). Cell lines were infected with the pseudotyped retrovirus expressing ACE2. At 24 to 48 h after lipofection or 72 h after retrovirus infection, cells were infected with SARS-CoV (MOI = 1). Supernatant was harvested at the indicated times postinfection for analysis by plaque assay.

Expression of ACE2 from at least one of the two plasmid vectors resulted in SARS-CoV replication in Hec1B, MRC-5, and 17Cl 1 cells, but not A549, 293T, or AK-D cells (Table 2). Expression in 293 cells from pcDNA3.1 may have resulted in very-low-level virus replication based on observed low, but stable, virus titers. ACE2 expression from the pseudotyped retrovirus resulted in SARS-CoV replication in all cell lines.
virus spread. When extrapolating in vitro virus growth permitted replication, supporting a hypothesis of hematogenous lines would support virus replication. We further speculated we expected that one or more examined lung epithelial cell replication.

be sufficient to prolong cell viability, but not to inhibit virus considered, an interesting possibility is that for MA104 cells, MA104 cells are capable of an interferon response to viral cells, but CPE in the MA104 cells was delayed by 24 h. Vero E6 expected, SARS-CoV replicated in Vero E6 cells as well as in two studying SARS-CoV replication and pathogenesis. As ex-

TABLE 2. SARS-CoV in cells expressing human ACE2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Transfection vector</th>
<th>Virus titer (log_{10} PFU/ml) at hpi:</th>
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<tr>
<td>293</td>
<td>pCAGGS</td>
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<td>293</td>
<td>pcDNA</td>
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<tr>
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</tr>
<tr>
<td>MRC-5 pcCAGGS</td>
<td>ND</td>
<td>ND 3.6</td>
</tr>
<tr>
<td>MRC-5 Retrovirus</td>
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</tr>
<tr>
<td>A549 pcCAGGS</td>
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<tr>
<td>A549 Retrovirus</td>
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<tr>
<td>BHK Retrovirus</td>
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REFERENCES


