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

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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 ($TCID_{50}$) 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-

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TABLE 1. Cells examined for support of SARS-CoV replication

Cell type	Species	Tissue	Morphology	Order, family	Titer[a]	Source
SAE[b]	Human	Airway	Epithelial	Primate, *Hominidae*	<0.7	A. Brasier, University of Texas Medical Branch, Galveston (UTMB)
AEC[b]	Human	Alveolar	Epithelial	Primate, *Hominidae*	<0.7	A. Brasier
HUVEC[b]	Human	Vein	Endothelial	Primate, *Hominidae*	<0.7	Cambrex Corp., East Rutherford, N.J.
HeLa	Human	Cervix	Epithelial	Primate, *Hominidae*	<1	Tissue Culture Core Facility, UTMB
CaCo-2	Human	Colon	Epithelial	Primate, *Hominidae*	>7	Tissue Culture Core Facility
293	Human	Kidney	Epithelial	Primate, *Hominidae*	<1	S. Watowich, UTMB
293T	Human	Kidney	Epithelial	Primate, *Hominidae*	<0.7	S. Makino, UTMB
HepG2	Human	Liver	Epithelial	Primate, *Hominidae*	4	C. Shih, UTMB
Hec1B	Human	Uterus	Epithelial	Primate, *Hominidae*	<0.7	S. Makino
A549	Human	Lung	Epithelial	Primate, *Hominidae*	<2	A. Brasier
MRC-5	Human	Lung	Fibroblast	Primate, *Hominidae*	<2	American Type Culture Collection, Manassas, Va. (ATCC)
HCT-8	Human	Rectum	Epithelial	Primate, *Hominidae*	<0.7	ATCC
HUVEC-C	Human	Vein	Endothelial	Primate, *Hominidae*	<0.7	ATCC
MA104	African Green monkey	Kidney	Epithelial	Primate, *Circopithecidae*	>7	R. Ramig, Baylor College of Medicine, Houston, Tex.
Vero E6	African Green monkey	Kidney	Epithelial	Primate, *Circopithecidae*	>7	ATCC
Vero	African Green monkey	Kidney	Epithelial	Primate, *Circopithecidae*	>7	ATCC
AK-D	Cat	Lung	Epithelial	*Carnivora*, *Felidae*	<0.7	ATCC
Mv 1 Lu	Mink	Lung	Epithelial	*Carnivora*, *Mustelidae*	>6	ATCC
A.P.	Mongoose	Skin	Fibroblast	*Carnivora*, *Herpestidae*	<0.7	ATCC
DBT	Mouse	Brain	Astrocytoma	*Rodentia*, *Muridae*	<0.7	S. Makino
NIH 3T3	Mouse	Embryo	Fibroblast	*Rodentia*, *Muridae*	<1	Tissue Culture Core Facility
MLg2908	Mouse	Lung	Fibroblast	*Rodentia*, *Muridae*	<1	ATCC
17Cl-1	Mouse		Fibroblast	*Rodentia*, *Muridae*	<0.7	S. Makino
V79-4	Hamster	Lung	Fibroblast	*Rodentia*, *Cricetidae*	1	ATCC
CHO	Hamster	Ovary	Epithelial	*Rodentia*, *Cricetidae*	<1	M. Holbrook, UTMB
PHEF[b]	Hamster	Embryo	Fibroblast	*Rodentia*, *Cricetidae*	<3	L. Perrone, UTMB
PK15	Pig	Kidney	Epithelial	*Artiodactyla*, *Suidae*	>7	ATCC
RK13	Rabbit	Kidney	Epithelial	*Lagomorpha*, *Leporidae*	<2	ATCC
R9ab	Rabbit	Lung	Fibroblast	*Lagomorpha*, *Leporidae*	<1	ATCC
TBF	Tupaia		Fibroblast	*Scandentia*, *Tupaiidae*	<2	R. Tesh, UTMB, and G. Darai, University of Heidelberg, Heidelberg, Germany

[a] Virus titer is shown as the log PFU per milliliter or log Vero E6 $TCID_{50}$ per milliliter. Positive results were confirmed by a second determination.
[b] Primary cells.

cation. Virus replication in Vero E6 and CaCo-2 cells was reported previously (4, 17). HepG2 human liver epithelial cells may have supported limited replication, based on observed titers of 10^4 $TCID_{50}$/ml 6 dpi. This was not surprising, as Huh-7 human liver cells were recently reported to support efficient replication (11). Based on their similar origin and morphology to Vero E6 cells, replication in Vero and MA104 cells was also expected. CaCo-2 cells were previously reported to support lytic infection of two SARS-CoV isolates: FFM-1 and Hong Kong (4). The disparate results may be due to the different SARS-CoV strain used in our experiments or divergent CaCo-2 lineages. This result merits further examination. Replication in Mv 1 Lu was also recently reported (11).

All of the cell lines that supported replication were epithelial in origin, though not all epithelial cells supported replication. The cell lines examined represented six orders from the class *Mammalia*. Permissive cell lines were derived from three different orders. Of the three cell lines examined that are derived from *Carnivora*, the same order as the putative reservoir, only one, Mv 1 Lu (family *Mustelidae*), supported virus replication. It is not yet clear whether SARS-CoV utilizes ACE2, CD209L, or some alternate receptor in all identified permissive cell lines. It was recently shown that murine ACE2 does not allow for efficient SARS-CoV replication, raising the possibility of alternate receptor use in nonprimate 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 2×10^7 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 and was maximal by 72 hpi. SARS-CoV growth in CaCo-2 cells was slower and less productive. Virus titers were maximal at ≥4 dpi. Virus titers of $>10^7$ $TCID_{50}$/ml were observed 6 dpi in the initial experiments (Fig. 1).

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

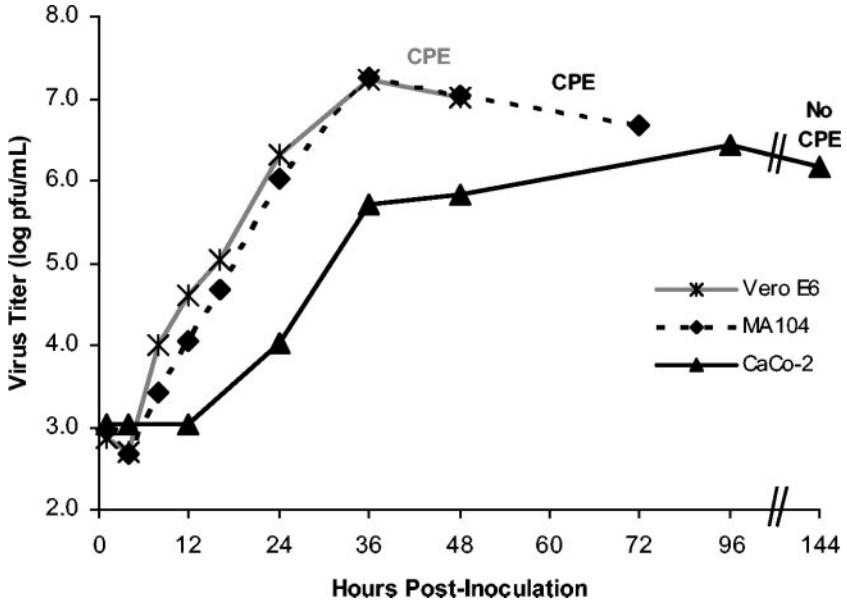

FIG. 1. Multistep growth curves for SARS-CoV in Vero E6, MA104, and CaCo-2 cells. Confluent cell monolayers were infected at low multiplicity (MOI = 0.001 to 0.005). The appearance of CPE in Vero E6 and MA104 cells is indicated. Confluent CPE is indicated by termination of the time course. CaCo-2 cells did not exhibit CPE.

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.

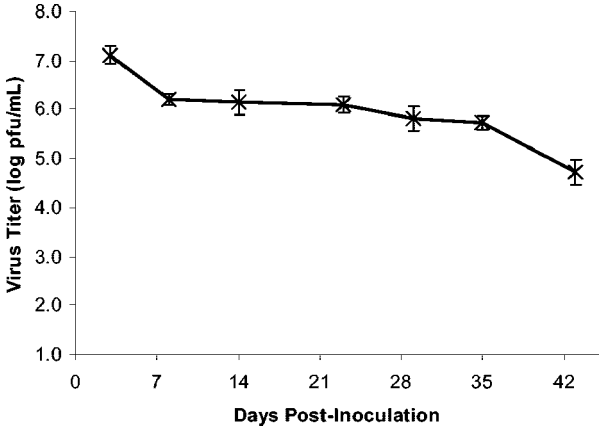

FIG. 2. Long-term maintenance of SARS-CoV-infected CaCo-2 cells. Confluent cell monolayers were infected at low multiplicity (MOI = 0.001 to 0.005; n = 7 to 8). At each time point, culture supernatant was removed, frozen for analysis, and replaced with fresh medium. CaCo-2 cells were passaged once (1:6 at 8 dpi).

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

TABLE 2. SARS-CoV in cells expressing human ACE2

Cell line	Transfection vector	Virus titer (log_{10} PFU/ml) at hpi:			
		0	24	48	72
293	pCAGGS	ND[a]	2.4	1.0	0.7
293	pcDNA	ND	2.9	3.2	2.9
293	Retrovirus	ND	5.0	5.8	6.3
293T	pcDNA	ND	2.3	2.0	1.9
293T	Retrovirus	4.4	4.6	5.6	5.3
Hec1B	pcDNA	ND	4.8	5.1	5.6
MRC-5	pCAGGS	ND	ND	ND	3.6
MRC-5	Retrovirus	ND	6.1	ND	5.7
A549	pCAGGS	ND	ND	ND	<0.7
A549	Retrovirus	ND	5.3	5.4	5.2
HeLa	Retrovirus	ND	4.6	5.2	3.9
17Cl 1	pCAGGS	3.7	ND	ND	4.0
17Cl 1	pcDNA	ND	4.4	4.9	5.0
AK-D	pCAGGS	ND	ND	ND	1.6
AK-D	Retrovirus	ND	6.3	ND	5.6
BHK	Retrovirus	ND	5.8	5.4	4.8

[a] ND, not determined.

examined, including those still refractory following plasmid ACE2 expression. Based on these results, the in vitro host range of SARS-CoV is primarily determined by the presence of its receptor, ACE2.

The 293T cells were unable to support virus replication after plasmid ACE2 expression despite high levels of ACE2 expression (data not shown). However, ACE2 expression from the pseudotyped retrovirus resulted in efficient virus replication in 293T cells. Similar observations were made in A549 and AK-D cells. Since ACE2 can be cleaved and secreted from the cell surface (7), it is possible that high levels of ACE2 expression from the plasmid vectors may have resulted in sufficient secreted ACE2 to block the infectivity of the inoculum and/or first-generation progeny. This observation and hypothesis correlate with a report that very high levels of ACE2 mRNA expression did not render colonic cell lines permissive to SARS-CoV infection (2).

This study was undertaken to identify common traits of cells found to be permissive or refractory to SARS-CoV infection in the hope of developing in vitro and in vivo model systems for studying SARS-CoV replication and pathogenesis. As expected, SARS-CoV replicated in Vero E6 cells as well as in two additional African green monkey kidney cell lines, Vero and MA104. SARS-CoV grew equally well in Vero E6 and MA104 cells, but CPE in the MA104 cells was delayed by 24 h. Vero E6 cells are known to be deficient in interferon production, while MA104 cells are capable of an interferon response to viral infection (6, 9, 23). While other cellular differences must be considered, an interesting possibility is that for MA104 cells, the cellular interferon response to SARS-CoV infection may be sufficient to prolong cell viability, but not to inhibit virus replication.

Based on the pathology of SARS-CoV infection in humans, we expected that one or more examined lung epithelial cell lines would support virus replication. We further speculated that primary HUVECs and/or the HUVEC-C cell line might support replication, supporting a hypothesis of hematogenous virus spread. When extrapolating in vitro virus growth permissiveness to in vivo pathogenesis, it is important to remember that different lineages of cell lines may have evolved in culture to display different phenotypes. This may account for the report that CaCo-2 cells support lytic replication of SARS-CoV (4), whereas in our hands, SARS-CoV caused nonlytic infection of CaCo-2. The report of growth in unmodified 293 cells (Spiegel and Weber, personal communication) but our failure to achieve growth without exogenous ACE2 expression, and the report of high levels of ACE2 expression in A549 cells (14) but our observation that retroviral ACE2 expression is required to alter A549 from refractory to permissive, could also be attributable to different lineages of cell lines.

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